PB86-149192

ANEUPLOIDY: ETIOLOGY AND MECHANISMS

U.S. Environmental Protection Agency
Washington, DC

Jan 86

U.S. Department of Commerce
National Technical Information Service

NTIS

PB86-149192

EPA/600/9-86/003
January 1986

ANEUPLOIDY

Etiology and Mechanisms

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TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO.
EPA/600/9-86/003

2.

3. RECIPIENT'S ACCESSION NO.
PB86-149192/AS

4. TITLE AND SUBTITLE
Aneuploidy: Etiology and Mechanisms

5. REPORT DATE
January 1986

6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)
Vicki L. Dellarco*, Peter E. Voytek*, and Alexander Hollaender°, editors

8. PERFORMING ORGANIZATION REPORT NO.

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Reproductive Effects Assessment Group (RD-689)
Office of Health & Environmental Assessment
Office of Research & Development
U.S. Environmental Protection Agency, Washington, DC 20460

10. PROGRAM ELEMENT NO.

11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS
Office of Health and Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC 20460

13. TYPE OF REPORT AND PERIOD COVERED

14. SPONSORING AGENCY CODE
EPA/600/21

15. SUPPLEMENTARY NOTES
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16. ABSTRACT

The "Symposium on Aneuploidy: Etiology and Mechanisms" was held from March 25-29, 1985. This Symposium developed as a consequence of the concern of the Environmental Protection Agency with the support of the National Institute of Environmental Health Sciences about human exposure to environmental agents that cause aneuploidy. These proceedings of the conference contain what is currently known about the underlying causes, the origins, and the extent of the problem of human aneuploidy, and whether exposure to environmental agents is associated with an increased incidence of aneuploidy in humans. Basic research findings in the area of mitosis and meiosis are also presented and related to possible mechanisms of how aneuploidy may be produced spontaneously and chemically. A survey of data regarding the chemical induction of aneuploidy in experimental organisms is presented. Outstanding scientists from different fields were invited to cover a broad perspective of aneuploidy from the molecular aspects to the human situation.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT
Release to the public

19. SECURITY CLASS (This Report)
Unclassified

20. SECURITY CLASS (This page)
Unclassified

21. NO. OF PAGES
556

22. PRICE

EPA Form 2220-1 (9-73)

The symposium at which the papers reproduced in this volume were presented was funded by the United States Environmental Protection Agency and the National Institute of Environmental Health Sciences under contract numbers DW89930860-01-0 and RW75931350-01-0. Although the manuscript has been subjected to Agency review and approved for publication, it does not necessarily reflect the views and policies of the Agency and no official endorsement should be inferred. The opinions expressed within respective chapters reflect the views of the authors, and mention of trade names or commercial products does not constitute endorsement by the Agency or recommendation for use. The discussion comments after most presented chapters have been edited for clarity and brevity.

PREFACE

The "Symposium on Aneuploidy: Etiology and Mechanisms" was held at the Carnegie Institution of Washington Auditorium from March 25-29, 1985. This Symposium developed as a consequence of the concern of the Environmental Protection Agency with the support of the National Institute of Environmental Health Sciences about human exposure to environmental agents that cause aneuploidy. The program was chosen to explore what is currently known about the underlying causes, the origins, and the extent of the problem of human aneuploidy, and whether exposure to environmental agents is associated with an increased incidence of aneuploidy in humans. Basic research findings in the area of mitosis and meiosis were presented and related to possible mechanisms of how aneuploidy may be produced spontaneously and chemically. A survey of data regarding the chemical induction of aneuploidy in experimental organisms was presented. Outstanding scientists from different fields were invited to cover a broad perspective of aneuploidy from the molecular aspects to the human situation.

We hope that the publication of the proceedings will share the enthusiasm of the meeting and its scientific content. The topic of aneuploidy has received little attention and it is the purpose of this Volume to establish a scientific basis for assessing health risks posed by environmental exposures to aneuploidy-inducing chemicals.

Vicki L. Dellarco
Peter E. Voytek
Alexander Hollaender

ACKNOWLEDGEMENT

The Editors of the proceedings of the "Symposium on Aneuploidy" wish to acknowledge the support of Dr. Elizabeth L. Anderson of the Environmental Protection Agency.

Dr. Anderson is an internationally recognized leader in the field of risk assessment. Since coming to the EPA in 1971, she has directed her energies toward the protection of the public from environmental toxicants. She was instrumental in establishing the Agency's Carcinogen Assessment Group and served as its Executive Director from 1976 to 1979. Dr. Anderson pioneered the EPA's efforts in risk assessment. Under her current leadership as Director of the Office of Health and Environmental Assessment, the EPA has proposed risk assessment guidelines in five areas: carcinogenicity, mutagenicity, exposure, developmental toxicants, and complex mixtures. For her innovative efforts in the area of risk assessment, Dr. Anderson was awarded a Gold Medal, the highest honor an Agency employee can receive.

A writer of several papers and a well-known public speaker on risk assessment and related scientific issues, Dr. Anderson was a member of the Steering Committee, which established the Society of Risk Analysis, and currently serves as its President. She continues to encourage the scientists in her office to seek and develop new methodologies for conducting health risk assessments, as is demonstrated by these proceedings on aneuploidy.

Organizing Committee: James Allen, Vicki Dellarco, Frederick de Serres, Richard Hill, Alexander Hollaender, Michael Shelby, Raymond Tice, Peter Voytek, Michael Waters, and Claire Wilson; Scientific Advisory Committee: B.R. Brinkley, Ernest Hook, T.C. Hsu, Montrose Moses, and Liane Russell.

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INTRODUCTION: ASSESSMENT OF HEALTH RISKS

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I would like to provide first a very brief overview of risk assessment and, second, the reasons why regulatory agencies like the U.S. Environmental Protection Agency (EPA) are interested in chemicals that cause aneuploidy.

Regulatory agencies like EPA are given congressional mandates to protect human health and the environment. Risk assessment is an important part of this process that is illustrated in Fig. 1, which shows the components that go into making a regulatory decision. Risk assessment involves identifying an agent as causing an adverse effect, determining how bad that effect is, and estimating the incidence of adverse effects in a given population. Then, depending upon the particular legislation, the costs and benefits of reducing environmental levels are weighed before making a regulatory decision. The decision could range from a complete ban of the chemical to no control at all. Risk assessment is a critical tool in the regulatory process for discriminating among environmental health problems, without which regulation to control chemicals in the environment could not occur. Unlike many laboratory situations we do not deal with scientific absolutes, but instead are working in a legal framework and often times have to make judgments based on the toxicological data available to us. Because of this, decisions regarding human health risk may be made with minimal data to maximal data, as illustrated in Fig. 2. Of course, the better the data the better the assessment that can be made.

For example, under Section 5 of the Toxic Substances Control Act (TSCA) dealing with new chemical substances of which approximately 1,200 are reported yearly to EPA, acute studies and possibly a bacterial gene mutation test may be available. Yet a decision as to whether these chemicals should enter the environment and what precautions should be taken must be made. Of course, structure activity relationships play an important role in these assessments in which data are limited. Under "maximal data," we may have 2-year chronic exposure studies in 2 mammalian species and possibly human epidemiology studies in order to make an evaluation. However, it usually is not enough simply to say a chemical may be a potential carcinogen, teratogen, or mutagen to humans. This was quite evident in the Occupational Safety and Health Administration's (OSHA) hearings on benzene (5). OSHA, in the late 1970s, wanted to lower the permissible exposure levels in the workplace from a standard of 10 ppm to a standard of 1 ppm stating that the number of cancers prevented by this action "may be appreciable," that "the benefits of the proposed standard are likely to be

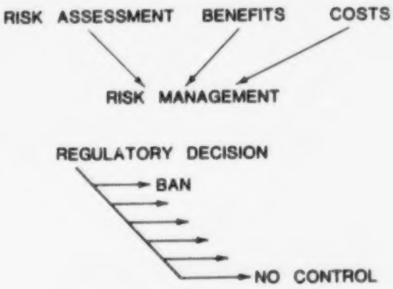

Fig. 1. Process of regulatory decision making in risk assessment.

appreciable," and that the "substantial costs for the new standard are justified in light of the hazards." OSHA felt that the quantitative estimates of risk for cancer were too imprecise to provide numbers of people who would benefit by not getting leukemia by reducing the standard 10-fold.

The Court of Appeals found them insufficient because OSHA failed "to estimate the extent of expected benefits." The court would have required OSHA to supply a specific numerical estimate of benefits derived through mathematical techniques for "risk quantification" or "cost-effectiveness analysis." This finding was appealed to the Supreme Court which upheld the lower court's decision on July 2, 1980 and questioned "OSHA's determination that available quantification techniques are too imprecise to permit a reasonable numerical justification of risk." Therefore, in hindsight it would seem that OSHA's case would have been stronger if the reduction in risk by lowering the standard from 10 to 1 ppm had been estimated.

Health risk assessments are conducted in the following manner (Fig. 3). All available data on a chemical are critically examined with respect to adverse responses such as cancer, mutagenicity, developmental toxicity, and reproductive toxicity. A qualitative assessment is made and, if necessary and where possible, a quantitative assessment is made. Quantitative assessments may involve high-to-low exposure extrapolations, species-to-species extrapolations, selection of appropriate models, and association of levels of human exposure to the chances of disease contraction or toxic effects.

For endpoints such as cancer and heritable genetic diseases caused by gene mutations, there is sufficient biological justification to support the concept that there is no exposure level below which no adverse effects will occur. With this assumption, models can be selected to estimate disease

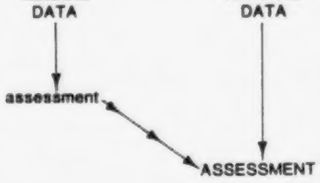

Fig. 2. Comparison of risk assessment derived from minimal data and maximal data.

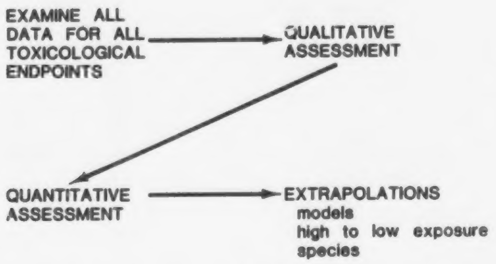

Fig. 3. Sequence of health risk evaluation.

incidences or adverse effects. The more we know about the mechanism of an adverse response, the better the selection of a model and, therefore, estimates of risk. For example, we know that one base change can lead to a mutation, or one "hit" resulting in a base change can lead to a mutation. For gene mutations, a theoretical dose-response curve model described by a linear equation may be chosen as shown in Fig. 4 (left graph). This model is verified by real data obtained for Drosophila exposed to ethyl methanesulfonate where sex-linked recessive gene mutations were scored vs target dose. On the other hand, a linear model may not be appropriate for chromosomal aberrations. At least 2 hits are necessary for structural mutations, like reciprocal translocations, to occur and a theoretical dose-response curve (Fig. 5, left graph) as described by a second-order equation may be appropriate. Actual data (Fig. 5, right graph) on Drosophila exposed to ethyl methanesulfonate, where reciprocal translocations are scored vs target dose, show the actual curve to be nonlinear, involving multiple hits. These data were taken from the work of Dr. William Lee at Louisiana State University (4). Admittedly, factors such as repair and differential cell-stage sensitivity to mutation and killing are all important to the overall shape of the dose-response curve.

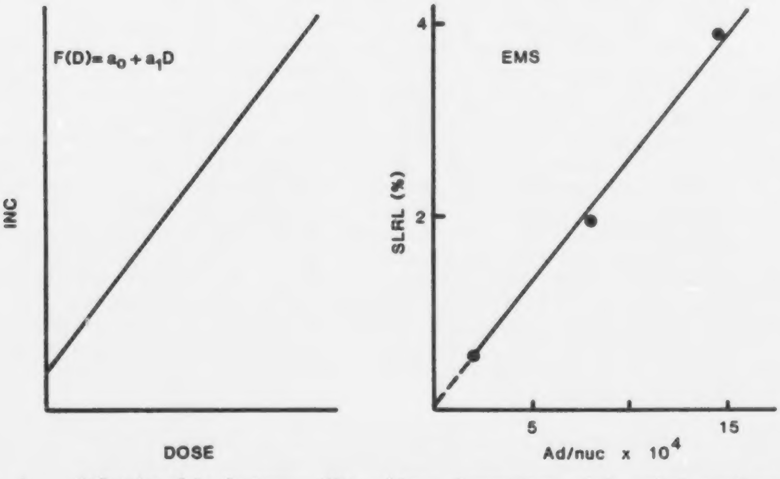

Fig. 4. Relationship between DNA adduct formation with ethyl methanesulfonate and induced frequency of sex-linked recessive lethals in *Drosophila melanogaster*. Adapted from Lee (4). D = exposure; a_0 and a_1 are constants.

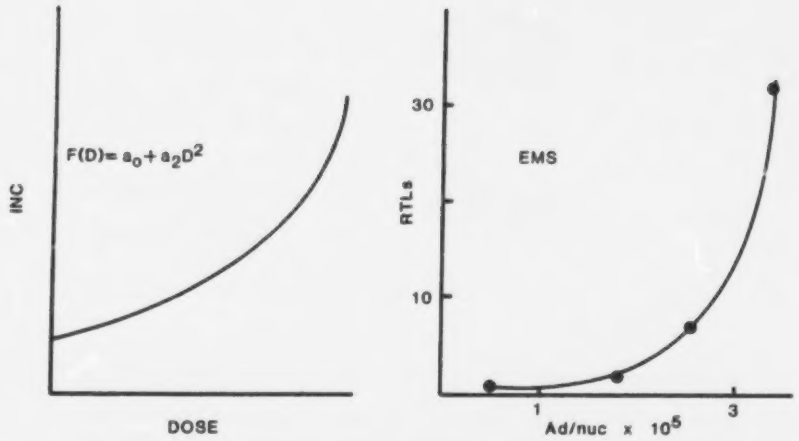

Fig. 5. Relationship between DNA adduct formation with methanesulfonate and reciprocal translocations in *Drosophila melanogaster*. Adapted from Lee (4). D = exposure; a_0 and a_2 are constants.

To provide consistent approaches in conducting risk assessment, the EPA has recently proposed guidelines in 4 areas, one of which deals with mutagenicity. These risk assessment guidelines were proposed in the *Federal Register* on November 23, 1984 for public comment. The mutagenicity guidelines mainly focused on gene mutations and structural mutations but not numerical chromosomal abnormalities (e.g., aneuploidy). The guidelines did not address numerical chromosomal abnormalities in depth because of the lack of well-validated test methods and limited understanding of the underlying mechanisms of nonaneuploidy as induced by chemicals.

While the risk assessment guidelines were being developed, the EPA was also considering taking action on the widely used pesticide, benomyl. In 1979, no cancer data were available for benomyl and most actions taken by the EPA have, historically, been based on cancer. The mutagenicity data for gene and structural mutations indicated that benomyl was not mutagenic or at most was a weak mutagen; however, there were some studies that showed benomyl and its metabolite, methyl 2-benzimidazole carbamate, produced aneuploidy (8). Since little was known about assessing human risk to exposures of chemicals that induce aneuploidy, it was decided to obtain additional studies for cancer, teratogenicity, and gene mutations. No additional studies were sought on aneuploidy. Results of these tests are now available: the gene mutation tests were reported as negative, whereas the cancer and teratology studies were positive (7). The EPA's final action most likely will be based primarily on these toxicological endpoints. The proposed EPA position document on benomyl concludes that since little is known about mechanisms of how aneuploidy is induced and, given the uncertainties in extrapolations from nonhuman test systems to human exposures, this endpoint should not presently be a consideration in setting the health standard.

The Reproductive Effects Assessment Group took on the crusade to address the issue of potential health risks caused by exposure to agents that cause aneuploidy. We learned that the National Institute of Environmental Health Sciences (NIEHS) sponsored a workshop in late 1978 on "Systems to Detect the Induction of Aneuploidy," which was headed by Dr. Frederick de Serres (6). The purpose of this effort was to review aneuploidy and to assess the systems used to detect aneuploidy, and it was apparent that short-term test systems needed to be developed to screen for compounds that induce aneuploidy.

More recently, in late 1983, the Reproductive Effects Assessment Group organized a small workshop to explore further the progress of test method development for the detection of aneuploidy, and to assess the current molecular processes of how aneuploidy may arise. The workshop included participants from the EPA, the NIEHS, the Food and Drug Administration, the national laboratories, and some leading academic scientists conducting research on aneuploidy. The meeting was quite successful, and it gave regulatory scientists an opportunity to convey their needs for assessing health risk to the researchers in the field. The proceedings of this workshop will be published in 1985 in the journal Environmental Mutagenesis (3).

This same workshop led to the evaluation of critiques for aneuploidy test systems and a reassessment and update, by a series of working groups of investigators experienced in the field, of the scientific literature for classifying chemicals that induce aneuploidy. These critiques and classifications will be published as an issue of the journal Mutation Research in late 1985 (2).

The goals of the workshop were to provide guidance to assess the potential risk of human exposure to chemicals that cause aneuploidy, and to convey to researchers in this field the need to focus on health risk assessment and the research that is needed to better our methods of estimating human risk from exposure to chemicals that cause numerical aberrations. In the past our office also conducted a workshop on "Assessment of Risks to Human Reproduction and to Development of the Human Conceptus" (1). The goals of this workshop were very similar to the goals just described.

However, it was obvious that a broader, more in-depth meeting was needed. The enthusiasm of the participants along with the prescience of Dr. Alexander Hollaender led to this larger international symposium.

In summary, we have brought together speakers and discussants of different disciplines of science to cover a broad perspective of aneuploidy from molecular aspects to the human situation to talk about the mechanisms of aneuploidy and the consequences of human exposure to chemicals that cause aneuploidy, in order to assess their risk to people.

This symposium motivated and was in part responsible for the EPA's recently proposed Guidelines for the Health Assessment of Suspect Developmental Toxicants, as well as for the reproductive toxicity guidelines which are currently being written. I anticipate that this publication will also markedly affect future risk assessments on chemicals that cause numerical chromosomal abnormalities.

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Editors' Comment: See also the publication entitled *Polyploidy: Biological Relevance* edited by Walter H. Lewis (Plenum Press, New York, 1980) based on the May 24-27, 1979 proceedings of the international conference held at Washington University, St. Louis, Missouri.

THE IMPACT OF ANEUPLOIDY UPON PUBLIC HEALTH: MORTALITY AND MORBIDITY ASSOCIATED WITH HUMAN CHROMOSOME ABNORMALITIES

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INTRODUCTION

The rationale for a conference on the etiology and mechanisms of aneuploidy is of course based upon the well-recognized association of chromosome abnormalities with human morbidity and mortality. Down syndrome, the best recognized phenotype, is only one of numerous adverse consequences of aneuploidy.

This review of the morbidity and mortality of chromosome abnormality indirectly provides a rationale for application of scientific and technological resources to a search for the causes of aneuploidy, and implicitly, to methods of primary prevention. Discussions of economic costs appear elsewhere in subsequent chapters (Conley; Edel; this Volume).

The classical definition of aneuploidy or of the aneuploid state (Tackholm, 1922; cited by Rieger et al. [48]) is a deviation from a multiple of the monoploid state, which in humans is 23. Thus the presence of an extra chromosome or the absence of a chromosome (normally present) is classified as aneuploidy. Triploidy (-69) or tetraploidy (-92), however, is not so classified. More recently, the definition has been broadened by Dyer et al. (1970; cited by Rieger et al. [48]) who have defined segmental or partial aneuploidy as the presence of extra segments or the absence of chromosome segments normally present, a result of chromosome structural rearrangement without necessarily a gain or loss in the total number of chromosomes or centromeres. Today the unmodified term, "aneuploidy," is somewhat ambiguous. It is used sometimes to refer to both a numerical or a structural abnormality, and at other times in its original meaning only.

Moreover, the term, "aneuploidy," is sometimes confused with "heteroploidy" which encompasses all numerical chromosome abnormalities, including triploidy and tetraploidy.

The concern of this Symposium has been defined by its sponsors as aneuploidy in the more restrictive, original definition. Nevertheless, it does not appear appropriate in consideration of the impact of chromosome abnormalities to exclude structural rearrangements or triploidy and tetraploidy. In the material that follows I will consider these types of abnormalities as well, making estimates of their relative impact where data are available.

---

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Chromosome abnormalities may also be classified as to whether they are of germinal or somatic origin. In view of recent knowledge, however, it would seem more appropriate to define 3 stages: prezygotic, zygotic, and postzygotic. Table 1 presents timing of origin associated with numerical abnormalities and related phenomena (see below). Further discussion on investigation of the origin of abnormalities also appears in a subsequent chapter (Hassold, this Volume).

Most numerical chromosome abnormalities are usually presumed to have a prezygotic "origin." (The exceptions are mosaic 45,X lines, triploidy, tetraploidy, and situations in which numerical abnormalities occur in only a single tissue, as for example in many malignancies.) For autosomal trisomies at least, it is often difficult, however, to exclude formally the

Tab. 1. Numerical abnormalities by stage and type of origin.

Stage of origin	Type of origin	Outcome
Prezygotic	Meiotic nondisjunction	Trisomy Monosomy
	Anaphase [illegible]	Monosomy
	Meiotic failure	Triploidy[a] XX moles[c] XX (teratoma)
Zygotic	Dispermy	XX/XY Triploidy
	Cell fusion	Tetraploidy[d]
	Parthenogenesis[e]	XX (teratoma)
Postzygotic	Mitotic nondisjunction[f]	Trisomy[f] Monosomy (Mosaicism)[f]
	Anaphase loss[b]	Monosomy (Mosaicism)[f]
	Cell fusion	Tetraploidy[d]

---

[a] A diploid gamete presumably resulting from complete failure of segregation.
[b] Or loss of chromosomes at other stages.
[c] The reciprocal products are lethal.
[d] Whether tetraploidy is zygotic or postzygotic is unknown.
[e] Parthenogenetic development beyond a certain stage may occur or be induced by "fertilization" by an empty sperm.
[f] The exact proportion of recognized trisomy or 45,X monosomy resulting from postzygotic events is unknown.

possibility that they have resulted postzygotically through mitotic nondisjunction early in the development of the organism (see below). Conventionally, trisomies (except rare mosaics such as 45,X/47,XXX or 45,X/47,XXY) and nonmosaic 45,X monosomics are assumed to have resulted from primary meiotic nondisjunction. Secondary nondisjunction (i.e., "inheritance" of trisomy for an ostensibly normal patient who is a cryptic mosaic carrier of a trisomic line) also cannot usually be excluded in the case of autosomal trisomy.

The ratio of recognized structural abnormalities to numerical abnormalities is about 1 to 30 in recognized embryonic and fetal deaths, but is about 1 to 4 to 1 to 5 in livebirths (24,60). Estimates of the frequency of various categories of numerical and structural abnormalities appear in Tab. 2.

Further data on the prevalence of 3 different categories of structural abnormalities by origin and estimates of their phenotypic implications appear in Tab. 3. A summary of data from an interesting follow-up study of newborns with balanced rearrangements by Tierney et al. (57) appears in Tab. 4.

The phenotypic consequence of apparently balanced de novo rearrangements is probably a consequence of either interruption of gene sequences at the breakpoints of the rearrangements or undetected small deletions or duplicating, among other possibilities. Attempts using high resolution

Tab. 2. Rates per 1,000 of various categories of chromosomal abnormalities in livebirths. Slightly revised from data in Hook and Hamerton (24). These data are based upon liveborn surveys in several developed countries done between 1967 and 1974. They were done before prenatal diagnosis and selective amniocentesis were carried out. Banding studies, in general, were not used. Since the time of these studies the livebirth prevalence of numerical abnormalities has probably dropped about 25% because of amniocentesis and/or diminished childbearing by older women.

Category		Chromosomal abnormality	Rate (per 1,000)
Numerical	Autosomal (1.50)	47,+21	1.30
		47,+18	0.12
		47,+13	0.05
		Other	0.02
	Sex chromosome (1.75)	47,XXY	0.50 (0.93 in males)
		47,XYY	0.50 (0.93 in males)
		Other in males	~0.20 (0.40 in males)
		45,X	0.05 (0.10 in females)
		47,XXX	0.50 (1.00 in females)
		Other in females	0.20 (0.40 in females)
Structural	Balanced (1.95)	Balanced Robertsonian	0.90
		Inversions	0.14
		Other	0.90
	Unbalanced (0.60)	Robertsonian	0.07
		Deletions	0.09
		Supernumerary	0.30
		Other	0.14
		Total:	5.80

Tab. 3. Structural chromosome rearrangement by category and their phenotypic implications. Unpublished preliminary observations in about 60,000 prenatal cytogenetic diagnoses (fetuses 16 to 24 weeks of age) in which there was no known cytogenetic risk factor aside from age, as reported to the New York State Chromosome Registry. Only supernumerary de novo abnormalities of the structural aberrations have any association with age. Studies involve banding, usually at about the 300- to 400-band level of resolution. Numbers include both mosaic and nonmosaic abnormalities. There would be selective mortality of those with unbalanced (nonsupernumerary) abnormality, perhaps 30%, subsequent to amniocentesis.

	Balanced rearrangement		Unbalanced				Total		
			Supernumerary		Other unbalanced				
	Mutant de novo	Inherited	Mutant de novo	Inherited	Mutant de novo	Inherited	Mutant de novo	Inherited	Both
Total observed[a]	59–75[b]	245–261	26–32[c]	15–21	25–39[d]	30–44	110–146	290–326	436
Approximate rate per 10,000 fetuses[a]	10–13[b]	41–44	4–5[c]	3–4	4–7[d]	5–9	18–24	48–54	73
Estimated proportion phenotypically abnormal (%)	10	<5[e]	10	<5[e]	70[f]	97			

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[a] Range in numbers observed because of uncertainties in origin of some cases.
[b] 15–16% are mosaic. Note that all mosaics classified as artifactual are excluded.
[c] 54–60% are mosaic. Note that all mosaics classified as artifactual are excluded.
[d] 59–64% are mosaic. Note that all mosaics classified as artifactual are excluded.
[e] Note 3–4% of all newborns have an abnormal phenotype irrespective of karyotype.
[f] Assuming the mosaics have little phenotypic significance.

Tab. 4. Balanced translocations. Calculated from data in Tierney et al. (57).

	Translocation	No. affected	IQ(s)[a]	Retarded	Malformed	IQ > average
De novo	Reciprocal	7	−0.86 ± 1.76	3/7	1/7	2/7
	Robertsonian	3	−0.37 ± 1.08	0/7	0/3	1/3
Inherited	Reciprocal	7	+0.76 ± 0.44	0/7	0/7	6/7
	Robertsonian	9	−0.10 ± 0.94	0/7	0/7[b]	2/7
	Other	6	+0.25 ± 0.65	0/7	0/7	4/7

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[a] Values are relative to controls.
[b] Two with behavior problems.

methods to detect small abnormalities in those with de novo balanced rearrangements have not been successful (46). Nevertheless, these recent advances in chromosome substructure analysis have reduced significantly the size of detectable lesions, and advances in diagnosis appear likely to continue. Thus, it is difficult to specify precisely the proportion of recognized conceptions with a structural abnormality. Some instances of Mendelian disorders and diseases of unknown etiology have been attributed recently to subtle, heritable, structural chromosome rearrangements (Tab. 5). With increasing resolution, more and more examples are likely to be

Tab. 5. Conditions recently discovered to be associated with a consistent structural cytogenetic abnormality.

Disorder	Reference	Mode of inheritance	McKusick number*	Cytogenetic abnormality
X-linked mental retardation (and megalo testes)	53	X-linked incomplete dominant	30957*[a]	fra X(q27)
Prader-Willi syndrome	37	? recessive	26400	del 15(q11q12)
Wilms tumor and aniridia	16	? recessive	27780	del 11p13
Retinoblastoma and retardation	15	dominant	18020*[b]	del 13q14
Langer-Giedion syndrome trichorhinophalangeal II (and ? I)	5,18	? dominant	15023	del 8q24.1
Multiple endocrine neoplasia-2A,B	3	dominant	17140*	del 20p12.2
Miller-Dieker syndrome (lissencephaly)	12	recessive	24720*	del 17p13
DiGeorge anomalad*	11,36	? dominant	18840	del 22(pterq11)
Chondrodysplasia-punctata (X-linked recessive type)	10	X-linked	—	del 22q11

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* Asterisk assigned by McKusick implying inheritance is definite. The absence of an asterisk implies that mode of inheritance is conjectural.
[a] As noted in the text the fragile-X(q27) site may be simply a gene "marker" and not a chromosome abnormality in and of itself.
[b] Retinoblastoma with del 13q14 is usually associated with mental retardation, but this is not the case for the "simple" dominant allele.

found. A small chromosome alteration may delete or interrupt a DNA sequence of a gene locus, of which a dominant allele is known which results in a specific locus mutation. (In fact, aside from base substitutions it may be impossible conceptually to rigorously separate specific locus mutations from very small chromosomal deletions or other structural alterations.) A chromosomal structural alteration also may unmask a recessive disorder if the normal chromosome homolog carries the allele for the disease. Also, small structural chromosome lesions may produce a constellation of defects resulting in phenotypes whose etiology was previously unrecognized. Examples here are Prader-Willi syndrome and the Di George anomalad, although the latter probably has other causes besides a chromosome anomaly. Lastly, it may be noted that some specific locus mutations may produce structural chromosome "aberrations." One possible example is the fragile-X(q27) which is associated with mental retardation and large testes. It is not clear if the fragile-X site is (a) a "marker" of a specific locus mutation on the X chromosome which produces the syndrome, or (b) directly related to the pathogenesis of the syndrome in that the deleted X in brain cells and elsewhere results in the associated signs and symptoms. At the present time I believe it best to classify this condition not as a chromosome abnormality, but rather as a X-linked "allele," one of whose manifestations is a structural chromosome alteration, i.e., a fragile-X(q27). An analogous situation occurs with association of premature separation of the chromatids with Roberts syndrome (also known as the SC or pseudothalidomide syndrome), an autosomal recessive condition characterized by severe limb abnormalities. (Another condition has also been reported with this same association (62).

The magnitude of postzygotic, numerical chromosome abnormalities cannot be readily evaluated. Mitotic nondisjunction, a somatic event, is extremely difficult to infer with certainty, although it may well account for a significant proportion of disorders now classified as attributable to meiotic nondisjunction, particularly those instances classified as resulting from meiotic 2nd division nondisjunction. For example, mitotic nondisjunction in the early embryo might result in 47,+21 and 45,-21 lines. The 45,-21 line might die out, leaving 47,+21 as the predominant, if not exclusive, pattern and resulting in a child with classical Down syndrome. Parental origin studies could be consistent with 2nd meiotic division origin, but there would be no other grounds to suspect postzygotic origin. In general, numerical abnormalities of somatic origin (aside from malignancy) can now be inferred with a high degree of likelihood only from observations on individuals with mosaicism associated with presumptive anaphase loss, i.e., in Turner syndrome associated with the XX/XO genotype, and in male pseudohermaphrodites with XY/XO or related genotypes. Estimating the prevalence of such cases is difficult because the detection of the second line varies with the number of cells counted and the number of tissues evaluated. (For discussion, see Ref. 23 and 25.) Moreover, the admixture of cell lines, particularly in peripheral lymphocytes, may change with time. Mitotic nondisjunction and other somatic chromosome mutations occurring early in embryogenesis are likely to have more serious phenotypic consequences for development than those occurring later in life.

Postzygotic chromosomal aberrations in later life clearly make a major contribution to malignancy (Evans, this Volume). For other types of morbidity and mortality the contribution of such events is speculative. For example, with regard to aging, there is a clear increase of XO cells in peripheral lymphocytes with age, as well as evidence for other somatic-numerical changes (14,19,21; see also Ref. 50 on changes with age of sister-chromatid exchange rates induced by mutagens). The proportion of XO cells that accumulates with age is greater in females than in males. One may also speculate about the significance of such changes for autoimmune phenomena, which increase with age and are more prevalent in females. But despite these suggestive changes, the precise phenotypic significance of these findings remains unknown. I will consider postzygotic chromosome abnormalities further if their consequences are the same as those of prezygotic abnormalities (and if they cannot be distinguished etiologically).

EMBRYONIC, FETAL, AND INFANT MORTALITY: RATES OF ABNORMALITY IN CONCEPTUSES AT VARIOUS GESTATIONAL STAGES

Extensive work by Martin as well as Brandriff (Martin, this Volume) indicates that the overall rate of detectable cytogenetic abnormality in human sperm is about 8-9%. (This figure may be subject to idiosyncratic variation among subjects.) It seems likely that there are at least as many chromosome abnormalities in ova as in sperm. Indeed, judging from data on the origin of 47,+21 (Hassold, this Volume), there may well be more in ova.

Assuming this, and assuming that the sperm studied by the technique used by Martin are representative of those that fertilize human ova, one may estimate that the prevalence of human zygotes with chromosome anomalies is at least 16%, and probably much higher. Yet 3 weeks after conception (at 5 menstrual weeks) the estimated prevalence of chromosomally abnormal conceptuses is 5-10% (see below). This implies a significant loss of conceptuses with cytogenetic abnormality between the time of conception and the first clinical recognition of pregnancy. Such cases may include 47,+1 and autosomal monosomies which have not been reported yet in recognized embryos and fetuses.

In spontaneous abortions of embryos and fetuses, Warburton et al. (60) have summarized data on chromosome abnormalities from 6 studies (Tab. 6 and 7). These studies go up to age 28 weeks of gestation. Other studies discussed further below indicate a rate of about 6% cytogenetically abnormal in "stillbirths," i.e., fetal deaths 28 weeks or older. Using this information, and data on the estimated total number of embryonic and fetal deaths at various gestational intervals, I estimated that the proportion of such deaths with cytogenetic abnormality on all recognized embryonic and fetal death was about 30%. These data, in conjunction with the rate of abnormality in livebirths (24), resulted in an estimate of 5% abnormality in all recognized conceptuses, with a steady fall in prevalence rate through gestation (Tab. 8).

Cytogenetic studies of induced abortions done in early pregnancy provide data with which to compare these indirect estimates. Yet there is great variability in such studies. Yamamoto and Watanabe (63) summarized data from several different such reports. There is great variation, which is difficult to interpret completely, but may be related in part to differences in such variables as the population undergoing abortion, selection biases, gestation length, and whether or not incomplete specimens, i.e., those without detectable fetal tissue, were included.

The significance of the latter variable is illustrated by the results from an excellently described Swiss study in which there were 14/858 (1.6%) chromosome abnormalities among complete specimens and 9/52 (17.3%) abnormalities among the incomplete specimens (34). (Among 728 abortuses in which both types were collected, 52 [7.1%] were "incomplete" and 676 [92.9%] were "complete.") The results of this series of induced abortuses in which both complete and incomplete specimens were studied provide, I believe, some of the most reliable data available on the rate of abnormality in conceptuses by gestational interval (Tab. 9). The series included more women under 20 and 35-and-over than expected from Geneva livebirth data, which would tend to bias the results to (slightly) falsely high values, as the highest rates of abnormalities were in these two groups.

Tab. 6. Cytogenetic abnormality and embryonic and fetal death by gestational age.

	Gestational age in completed weeks					
	5-7	8-11	12-15	16-19	20-27	All known (pooled)
Proportion abnormal among those karyotyped[a]	24/137	488/965	625/1330	156/475	49/456	1342/3363
Percent abnormal[a]	17.5	50.6	47.0	32.8	10.7	39.9
95% confidence interval[b]	12.4-23.8	47.7-53.5	44.6-49.4	29.2-36.7	8.4-13.4	
Range (in percent) among studies	14.0-25.8	40.4-64.4	39.1-56.2	18.5-69.6	6.6-27.0[c]	

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[a] Calculated from data on 5 specific studies (in Honolulu, Geneva, Hiroshima, London, and New York) presented by Warburton et al. (60).

[b] Assuming homogeneity among populations studied. In fact, the ranges suggest that, at least for the 16- to 19-week group, this is unlikely.

[c] Excluding extremes of 0 and 40% in 2 studies with only a miniscule number studied.

Tab. 7. Embryonic and fetal deaths to 28 weeks: type of chromosome abnormality. Calculated from data in Warburton et al. (60). One study not considered in Tab. 6 is included in this tabulation. Data are through 27 completed weeks of gestation, i.e., 195 days.

	XO (%)	Trisomy (%)	Triploidy (%)	Tetraploidy (%)	Gross structural abnormalities (%)	Combinations (and mosaics) (%)
Range in 6 studies	15.4-34.7	44.8-58.6	9.8-20.3	2.5-8.3	1.3-5.1	0.7-7.7
Mean in 1,499 studied	22.9	52.5	16.4	4.3	3.6	4.0

Tab. 8. Estimates of proportion of all embryos or fetuses with chromosome abnormality in conceptuses of pregnancies recognized by specified stage of gestation. Ages are in complete weeks. See Ref. 28 for calculation.

Gestational interval completed (weeks)	Fetuses with cytogenetic abnormality aborted in interval (% of total conceptuses)	% of conceptuses at given stage with cytogenetic abnormality
5-7	1.10	5.0
8-11	1.70	4.2
12-15	1.20	2.4
16-19	0.30	1.1
20-27	0.14	0.8
≥28	0.06 (stillbirths)	0.7
		0.6 (livebirths)
Total:	4.40	—
Livebirths:	0.60	

Tab. 9. Abnormal abortuses by menstrual age in a Swiss study. Calculated from data of Kajii et al. (34).

Gestational (menstrual) age interval (weeks)	Proportion abnormal (95% confidence interval)	
6-7	2/34 = 5.9% (0.7-19.7%)	
8-9	9/217 = 4.1% (1.9-7.7%)	19/435 = 4.4% (2.5-6.7%)
10-11	10/218 = 4.6% (2.2-8.3%)	
12-13	1/204 = 0.5% (0.01-2.7%)	1/246 = 0.5% (0.01-2.2%)
14-15	0/42 = 0.0 (0-8.4%)	
≥16	1/13 = 7.7% (0.2-36.0%)	

Tab. 10. Comparison of indirect estimates of cytogenetically abnormal conceptuses with direct estimates from study of induced abortuses.

Gestational (menstrual) age interval (weeks)	Indirect estimates[a] (life table analysis)	Direct observations[b] (induced abortuses)	95% interval
5-7	5.0%	5.9%*	(0.7-19.7%)
8-11	4.2%	4.4%	(2.5-6.7%)
12-15	2.4%	0.5%	(0.01-2.2%)

* Observations on 6 and 7 weeks only.

[a] Ref. 28.

[b] Ref. 34.

One can calculate that the overall rates of abnormality adjusted for such differences would be about 85% of the rates given in this series. (Unfortunately, Kajii et al. [34] did not stratify their data by maternal age and gestation length, which would allow more precise adjustment.)

There is gross agreement between indirect estimates based on life-table analysis (28) and direct estimates from induced abortuses in the study of Kajii et al. (34) (Tab. 10). The only serious disagreement is for the period 12-15 weeks in which a much lower rate of abnormality, 0.5%, is noted in the induced abortuses studied. This low rate is probably attributable to statistical fluctuation because it is about the rate detected in livebirths usually delivered at 40 weeks (24), in which one would expect a much lower rate of abnormalities than at 12-15 weeks.

The only other observers, to my knowledge, to provide data on induced abortions stratified by gestational age are Yamamoto and Watanabe (63) in a series from Japan. Their intervals are different from those of Kajii et al. (34) so it is not possible to compare the data precisely, but it is clear that they found higher rates at all gestational ages (Tab. 11).

The reasons for this difference are uncertain. They may reflect some difference between the Japanese and the Swiss populations in induced abortions or in the women seeking induced abortions. (Data from spontaneous abortuses suggest no major ethnic difference between Japanese and European populations in cytogenetic abnormalities; see Ref. 60.) Alternatively, some methodological difference is possible. For example, a low ascertainment rate of "incomplete" specimens by Kajii et al. would result in falsely low rates, although this appears unlikely in view of their definition of

Tab. 11. Comparison of data from 2 series on abnormalities in induced abortuses.

Gestational (menstrual) age interval (weeks)	Japan Yamamoto and Watanabe (63)	Switzerland Kajii et al. (34)
5-6	10/108 = 9.3%	—
6-7	—	2/34 = 5.9%
7-8	37/537 = 6.5%	—
8-9	—	9/217 = 4.1%
9-10	25/389 = 6.4%	—
10-11	—	10/218 = 4.6%
11-12	7/130 = 5.4%	—
12-13	—	1/204 = 0.5%

this problem and specific steps taken to avoid it. Another possible source of bias is "missed" abortions, which are retained spontaneous abortions usually expelled by operative means and thus classified as "induced." Kajii et al. reported about a 50% rate of cytogenetic abnormality in such "missed" abortions. Inclusion of just a few such events in a series of elective abortions would distort upwards the reported rate of abnormalities. This may account for the higher rate of abnormalities in the data of Yamamoto and Watanabe than in those of Kajii et al., as the former do not comment on this factor nor on attempts made to avoid it, whereas Kajii et al. do so specifically and report lower total rates of abnormality. The meticulous attention to biases ot various types by Kajii et al. (34) and their attempts to avoid them make the results of their study the presumptive benchmark until subsequent studies using the same methodology are performed.

Some types of patterns of embryonic and fetal death occur consistently in association with specific chromosome patterns. For example, benign cystic teratomas (dermoid cysts) have 46,XX patterns with all homozygous maternal markers (38) indicating failure of 2nd maternal meiotic division and absence of fertilization (or else fertilization by an anucleate sperm and subsequent parthenogenetic development). Moles (types of trophoblastic tumors) which are "complete," i.e., without fetal development, have primarily 46,XX patterns, with all homologous paternal markers, resulting presumably from fertilization of an anucleate egg by a haploid sperm whose component doubles or by a diploid sperm which resulted from failure of 2nd division segregation. A few moles probably result from double fertilization of an anucleate ovum in view of observed 46,XY patterns (43). There is evidence for ethnic variation in complete moles with a high rate in Orientals, particularly Filipinos (32). It is a moot question whether these outcomes associated with apparently normal complements, but originating from failure of chromosome segregation in meiosis, should be classified as a "cytogenetic abnormality." But partial hydatidiform moles, i.e., those associated with some fetal development, are usually associated with unequivocal abnormality: triploidy with 2 paternal and 1 maternal complements (32,55). More extensive relationships of cytogenetic abnormality to pathology associated with embryonic and fetal death have been described in detail by Byrne et al. (6).

Data on chromosome abnormalities in stillbirths and deaths in livebirths have been summarized by Sutherland et al. (54) and Angell et al. (1). Data from these two summaries appear in Tab. 12. As these indicate, macerated stillbirths, i.e., those which have been retained for some time after fetal death, have a much higher rate of abnormality than those which are "fresh," i.e., expelled immediately. And as evident in the data of Angell et al. (1) the presence of a recognized malformation makes the presence of a chromosome abnormality even more likely. The types of chromosome abnormalities observed in infant deaths are summarized by Angell et al. (1) and appear in Tab. 13. As may be noted most are numerical abnormalities, and 47,+18 is the most prevalent chromosome anomaly here.

The only pertinent data in nonhuman primates of which I am aware is a study by Small and Smith (51) who found no cytogenetic abnormality in 19 "stillborns" or 7 neonatal deaths in Macaques, a suggestive result, but not statistically significantly different from observations in humans.

BIRTH DEFECTS

Earlier, I estimated that 4-8% of all children with a "serious" malformation are affected because of a chromosome abnormality (29). (This is derived from assuming 1.6 per 1,000 livebirths have a serious malformation

Tab. 12. Percent chromosome abnormality in stillbirths ($\geq$28 weeks) and in liveborns subsequently dying.

From Sutherland et al. (54)	Stillbirths	Macerated	11.0%	(112)
		Nonmacerated	3.8%	(340)
		All	5.7%	(457)[a]
	Livebirths	Early neonatal deaths	5.0%	(824)
		Late neonatal deaths	5.8%	(86)
		Deaths 28 days to one year	6.9%	(203)
		Pediatric deaths	7.4%	(97)
From Angell et al. (1)	Macerated stillbirths	With malformation	42.9%	(21)
		Without malformation	2.6%	(114)
	Fresh stillbirths	Without malformation	0%	(61)
	Malformed	Livebirths who died (and fresh stillbirths)	7.7%	(91)
	Nonmalformed livebirths who died		0.9%	(213)

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[a] Of these, 5.2% have a numerical abnormality; 0.5% have a structural abnormality.

and a cytogenetic defect and 2-4% of all infants have a serious malformation.) This approach classifies the phenotype of Down syndrome as a malformation. If only those with morphological abnormalities per se are included, then the proportion is lower (around 3-5%) (29). But some categories of birth defects are associated with higher rates of involvement, for example, heart defects. I estimated roughly that 10% of all livebirths with (significant) congenital heart defects had a chromosome abnormality (29). At least some pediatric cardiologists expressed skepticism; they did not believe that the proportions were this high. I suspect this impression may have resulted from the high mortality of those with chromosome abnormalities, such as those with trisomy 18 and trisomy 13, as well as many of those with Down syndrome and heart defects. But recently, Ferencz (13)

Tab. 13. Chromosome anomalies in perinatal deaths. Calculated from Angell et al. (1).

Anomalies	Number of	
47,+21	16	85% numerical
47,+18	26	
47,+13	9	
45,X	2	
Other sex chromosomal abnormalities	10	
Triploidy	4	
Other (numerical)	11	
Structural abnormalities	14	15% structural
Total:	92	

reported from the Baltimore heart study that 111/833 (13.3%) (95% confidence interval of 10.8% to 15.8%) infants with congenital heart defects had a chromosomal abnormality, confirming that the rate estimated indirectly was not falsely high. In fact, if anything my earlier estimate was lower than the figure in this large-scale direct study.

Some interesting results have also appeared on the proportion of children with multiple malformations and retardation of unknown origin. Among studies at the Universities of Wisconsin and Tennessee by former students and fellows of K. Patau (see Ref. 56 for summary and references), 6.2% (44/710) of individuals with 3 or more independent, significant malformations and established mental retardation of unknown etiology had a chromosome abnormality, compared with a rate of 0.7% (5/710) in controls. (The proportion in controls is close to the rate of cytogenetic abnormality in consecutive livebirths of about 0.6%.) It is emphasized that these results exclude individuals with phenotypes of chromosomal "syndromes," such as Down syndrome, trisomy 13 syndrome, trisomy 18 syndrome, etc., that were suspected before study, as well as those with other recognized conditions of known etiology. Because of the wish to document retardation, infants under 6 months were excluded. Thus, infants with cytogenetic abnormalities so severe that they would have died in the first 6 months are not in this study. The results of this study are summarized in Tab. 14.

A slightly different study by Coco and Penchaszadah (9) in Argentina reported an even higher proportion affected, 42/200 (21%), in those with retardation and 3 or more malformations. However, they excluded only the Down syndrome phenotype (and cases of suspected Turner or Klinefelter syndrome confirmed by Barr body study). Thus, they included 4 cases of 47,+13 and 3 of 47,+18 among others, cases that were excluded from the previous investigation if the associated phenotype was evident. In addition, Coco and Penchaszadah included 11-12 newborns and very young infants in whom retardation was not yet proven, cases which were also excluded from the

Tab. 14. Abnormalities detected in 710 individuals without suspected syndrome but with mental retardation and 3 independent malformations (abstracted from Tharapel and Summitt [56]). The 5 abnormal controls had: 45,X/46,XX/47,XXY, inversions (2), 46/47,+mar, and a Robertsonian translocation. This was in 110 controls.

Numerical (7)		47,+18 47,XXX (n=2) 47,XYY 46/47,+E/92,XXYY 47,+D 47,+G
Numerical and Structural (8)		47,+der (n=2) 46,XX/47,+r (n=4) 47,+mar (n=2)
Structural	Apparently balanced (20)	inversions (n=11) Robertsonian (n=2) other (n=7)
	Unbalanced (19)	deletions (n=7) rings 1* other (n=11)*

* See also the category on both numerical and structural abnormality above.

series summarized by Tharapel and Summitt (56). The rate of abnormality in the Argentine series, if these instances are excluded, would still be about 12%, almost twice as high as the results of Tharapel and Summitt. But the very severe nature of the anomalies described among those studied by Coco and Penchaszadah suggests that there was a selective inclusion of the most deformed children in the Argentine study, contributing to the differences between this investigation and those of the Patau school.

RETARDATION AND OTHER BEHAVIORAL ABNORMALITIES

Studies of the epidemiology of mental retardation have indicated rates of gross detectable chromosome abnormalities in those with retardation of about 12% to 33%. I have reviewed those studies in detail elsewhere (29) and have discussed grounds for possible variation. These include changes in prevalence rates, particularly a fall in Down syndrome in recent years because of diminished childbearing by older women (see Ref. 27 for details on this point). Also, Down syndrome individuals are now less likely to be institutionalized in many jurisdictions leading to underascertainment of cases in studies limited to screening of institutions. At the same time I have the impression that mortality of Down syndrome cases has markedly fallen in recent years. The rate of detected chromosome abnormality also varies with IQ. In some early phenotypic studies in Canada and the U.S., the rates of Down syndrome were about 10% in those with IQs under 20, 34% in those with IQs 20 to 49, and about 3% in those with moderate retardation (39,41). (Latter figure, in particular, is subject to various biases based on both institutionalization patterns and selection of patients for study.)

The variation between studies in different populations may also relate to the variation in the actual proportion in the population with retardation. Among those with severe retardation, the data are not inconsistent with a rate affected of about 3 to 5 per 1,000. Among those with higher IQs, however, the data suggest a much wider range in the proportion affected. Thus, variation in the definition of who is retarded makes it difficult to estimate the contribution of cytogenetic abnormalities.

Some of these issues are illustrated by the results of a population study in Vasterbotten county of Sweden. Blomquist (4) reported 56 out of 161 cases (34.8%) with IQs under 50 with chromosome abnormalities and 8% in those with IQs greater than 50 (Tab. 15). These are underestimates because only those with suggestive stigmata had chromosomal investigation, so individuals with the most frequent sex chromosome abnormalities (47,XXY, 47,XXX, and 47,XYY) among others would not be detected. On the other hand, the proportion of livebirths to mothers 35 and over in this region, averaging about 13%, is about twice as high as that seen in many other areas at present and would result in prevalence rates of cytogenetic abnormality much higher than expected elsewhere. (In New York State, for example, the proportion is about 6-7%.) Perhaps the prevalence rates of chromosome abnormalities in the latter jurisdictions would be 50-70% of those given for this Swedish county, in a study conducted with the same methodology.

Results of another community survey, in Northeast Scotland by Innes et al. (31), appear in Tab. 16-18. This survey included 3,632 individuals with retardation in the original study group, of whom 3,020 were investigated intensively. The study included individuals of all ages with diagnosed retardation. Of those, 2,770, about 90% of the restricted group, had cytogenetic study. The proportion detected with abnormality was much lower than in the Swedish study (cf. Tab. 16 and 18), although a much broader range of abnormalities was discovered. This is the most extensive community survey known to me, but it probably underestimates the proportion of cytogenetic abnormality because of selective mortality.

Tab. 15. Chromosome abnormality and retardation in Vasterbotten county, Sweden, 1959-1970. Abstracted from Blomquist (4). Data are on children alive at age 1 yr.

		Year of birth			
		1959-1962	1963-1966	1967-1970	All years
Severe retardation (IQ < 50)	Down	42% (31/74)	23% (11/47)	28% (11/40)	33% (53/161)
	Other[a]	1% (1/74)	2% (1/47)	2.5% (1/40)	2% (3/161)
	Total	43% (32/74)	26% (12/47)	30% (12/40)	35% (56/161)
Mild retardation[b] (IQ 50-69)	Down[c]	—	—	—	6% (11/171)
	Other[c]	—	—	—	1% (2/171)
	Total	— (75)	— (43)	— (53)	8% (13/171)
Proportion of livebirths to mothers ≥ 35		16%	14%	10%	13.4%
Children born 1959-1970 and alive at one year of age		13,981	13,973	12,917	40,817

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[a] Two with 47,+18, one with 46,XX,13q+.
[b] Data by year of birth interval not given.
[c] One Down syndrome was a mosaic; other abnormalities were 46,XY,-22i(22q) and 47,+18.

Data are provided with regard to Down syndrome on 2 types of intelligence tests. (It is not clear what tests were used with regard to the other abnormalities, but it is implied that the WAIS test, suitably scaled for those of younger ages, was utilized.) About 15% of those with IQs 20 to 50 have Down syndrome, and somewhere between 1% and 5% in the IQs over 50 group do also. Depending upon which test was used, those with male sex chromosome abnormalities constitute about 2% of retarded males with IQs over 50, and almost 1% of retarded females with IQs in this range. It is also of interest that those with supernumerary chromosomes constitute almost 1% of those with IQs 20 to 50. The survey was unusual in that no examples of balanced translocations were reported, whereas about 6 would be expected based on observations in newborns. Smaller surveys, e.g., Jacobs et al. (32), find about 0.5% of balanced rearrangements in such studies.

With regard to the recently discovered fragile-X(q27) syndrome, as noted above it is uncertain if this is attributable to a chromosomal abnormality or to an allele at a locus tightly linked to the fragile-Xq27 site, in which case the site is simply a marker of the disorder. The livebirth prevalence of this condition in males is about 2 to 6 per 10,000 and perhaps 3 per 10,000 in females (58). Data on the proportion of males detected in one institution for retardation by phenotype appear in Tab. 19.

It is much more difficult to study mental dysfunction other than retardation. Those with other developmental disabilities, such as dyslexia, abnormalities of speech, and (minor) behavioral abnormalities, have to my knowledge not been investigated systematically. From prospective studies of infants with sex chromosome abnormalities, there is suggestive evidence that these occur more frequently in XXY and XYY children, although the evidence is variable and inconsistent (49,52). And as studies reviewed above suggest, those with minor structural cytogenetic abnormalities may also have a greater tendency to such outcomes without having gross mental retardation.

Tab. 16. Chromosome abnormalities in retarded in Northeast Scotland population. Abstracted and calculated from Innes et al. (31). 2,770 tested out of 3,020 in final cohort and 3,632 in original cohort.

Down syndrome			
47,+21	235		267
46/47,+21	10		
46,D/21 translocation	3	5	
46,G/21 translocation	2		
Not tested cytogenetically	17		
Deletions			
5p-	5		7
Other	2		
Supernumerary chromosomes	9		
Sex chromosome abnormalities			
Males (of 1,565)			
47,XYY	6		25
47,XXY	11		
46,XY/47,XXY	4		
48,XXYY	2		
48,XXXY	1		
48,XXXY/49,XXXXY	1		
Females (of 1,205)			
47,XXX	5		6
48,XXXX	1		
Total cytogenetically abnormal:	297 tested plus 17 with phenotypic diagnosis = 314 or 10.4-10.7% with abnormality.		

Behavior in adolescents and adults that leads to incarceration has been investigated extensively. One may define a "mental" institution as one for those with some abnormality of behavior, including retardation, a "penal" institution as one with restriction upon freedom because of security and/or legal and punitive requirements, and a "mental-penal" institution as one which meets both criteria. The proportions of those with one of four sex chromosome abnormalities in such settings and comparison with newborn rates appear in Tab. 20. Over 3%, i.e., more than 1/30, of all males in mental-penal settings studied to date have a sex chromosome abnormality. (These settings are for "criminal psychopaths," retarded criminals, criminals with behavior problems, etc., although not all in such settings may have been placed there appropriately, and conversely.) This strikes me as an extraordinarily high proportion. Nevertheless, it should also be recognized that some analyses suggest that only a relatively small proportion of those with XXY and XYY patterns eventually appear in either a penal or mental-penal setting (see Ref. 26 for further data and review).

ABNORMALITIES OF FERTILITY AND SEXUAL DIFFERENTIATION

Chromosome abnormalities also make a significant contribution to infertility and disorders of sexual differentiation. First with regard to

Tab. 17. Cytogenetic abnormalities by IQ in a Northeastern Scotland survey and comparison with distribution of total retarded population. Abstracted and calculated from Innes et al. (31).

	IQ<20	IQ:20-50	IQ>50	(IQ>70)	Not given	Total
Down syndrome (all genotypes)						
Test 1[a]	0 (23)[b]	153 (1,130)	76 (1608)	(5) (600)	38[d] (259)	267[d] (3,020)[b]
Test 2[c]	1 (2)[b]	152 (975)	6 (820)	(0) (134)	108[d] (1,223)	267[d] (3,020)[b]
Deletions[e]						
5p-	3	0	1	(0)	1	5
Other	0	2	0	(0)	(0)	2
Supernumerary[e]	0	7	0	(0)	2	9
Sex chromosome abnormalities[e]						
XYY	0	1	5	(0)	0	6
XXY	0	1	10	(0)	0	11
XY/XXY	2	1	1	(0)	0	4
Others in males	0	2	2	(0)	0	4
XXX	0	1	4	(0)	0	5
Other in females	0	0	0	(0)	1	1

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[a] Goodenough Harris Draw-a-person test.

[b] Results for entire population appear in parentheses. Thus out of 1,130 (in the 3,020) with IQ 20-50, there were 153 with Down syndrome. IQ distributions in those actually tested cytogenetically were not given.

[c] Wechsler adult intelligence scale (adjusted for children and adolescents).

[d] For reasons not clear a total of 239 with Down syndrome is listed in the original tables, including those not given, despite a prevalence listed elsewhere of a total of 267. The data on those "not given" listed in this table were derived by subtracting the results on those stated from the total of 267.

[e] It is not precisely clear what test results were used in assigning categories of IQ for these other classifications of cytogenetic disorders, although it appears likely it was the WAIS score. For evaluation of the results for the sex chromosome abnormalities, there were 1,693 males and 1,327 females in the group of 3,020. I estimate from the data given that the proportions of males with IQs in the <20, 20-50, >50, and >70 intervals were, respectively, 15.3%, 31.5%, 53.2%, and 14.6%, and for females, 14.3%, 39.0%, 46.7%, and 11.1%. (Note that the >70 group is included in the >50 group.)

fertility of males, the total proportion of those with chromosome abnormalities detected in blood studies in one study of about 1,600 men was 2.1% of which 1% were XXY (8). In studies of infertile men with sperm counts under 20 million per ml, the rate of cytogenetic abnormality was 3.5-6.1%, with at least half in each series with a 47,XXY pattern (8,22). In those with azoospermia, 15% had chromosome abnormalities, almost all of which were XXY (8). While the results on abnormalities detected in blood were consistent in the two studies, there were some puzzling discrepancies with regard to the results of testicular biopsies in men without chromosome abnormalities detected in the blood. The rate was 0.6% in the study of Chandley et al. (8) and 7.6% in the study of Hendry et al. (22).

There have been numerous cytogenetic studies of couples with infertility or diminished fertility because of multiple miscarriages. Over 50 such reports are cited by Pantzar et al. (44). In the pooled results of all of

Tab. 18. Chromosome abnormalities by location of retarded individuals in Northeast Scotland. Calculated from data in Innes et al. (31).

Location	47,+21 (and mosaics)	Translocation trisomy 21	Autosome deletions	Supernumerary markers	XXX	XXY (and mosaics)	XYY	Other	Total studied
Institutions for the retarded	72	0	2	2	1	4	0	1	734
Psychiatric hospitals	3	0	0	0	1	2	1	0	228
Community	162	5	4	6	3	9	5	4	1,704
Other (e.g., residential school)*	8	0	1	1	0	0	0	0	104
Total	235	5	7	9	5	15	6	5	2,770

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* Derived from frequencies given for the other three settings and the total.

Tab. 19. Fragile-X(q27) in a study of institutionalized, retarded males. Calculated from data of Froster-Iskenius et al. (17). Estimates of denominators in results on IQ are approximate.

Results by IQ	
<19	0/~12 = 0%
20-49	12/~120 = 10%
50-69	3/~41 = 7%
70-90	0/~20 = 0%
Unknown	0/49 = 0%
Results by phenotype and history	
Family history of retardation in males (consistent with X-linkage)	7/18 = 38.9%
Facial characteristics suggestive of syndrome	7/27 = 25.9%
Remainder	1/197 = 0.5%

these, the mean proportion of cytogenetic abnormalities detected per couple was 3.3% in those studied prior to 1970 and 6.3% subsequently. They suggest that use of banding techniques and, consequently, better detection of chromosome abnormalities are responsible for the difference. Yet there has been marked variation within "banding" studies, from detection rates as high as 13.4% by Mennuti et al. (40) to rates of 0% (structural) abnormality by Kardon et al. (35) and by Ward et al. (61). Probably this variability within banding studies is attributable to differences in ascertainment. Studies with very low rates may be those in which easily detected, noncytogenetic causes have not been excluded prior to cytogenetic study. This is also consistent with the results of Pantzar et al. (44), who in their own

Tab. 20. Sex chromosome abnormalities and presence in various institutions. Based on review in Ref. 26. All rates are sex-specific.

Table	XYY	XXY	XXYY	XXX (including mosaics)
Frequency in newborns	1/1,000 males	1/1,000 males	(?)1/50,000[a] males	1/1,000 females
Frequency in exclusively mental settings	3/1,000	4/1,000	0.7/1,000	3/1,000
Increased risk	3x	4x	35x	3x
Frequency in exclusively penal settings	4/1,000	4/1,000	none observed	4/1,000[b]
Increased risk	4x	4x	0[c]	4x[b]
Frequency in mental-penal settings	20/1,000	10/1,000	2/1,000	4/1,000[b]
Increased risk	20x	10x	100x	4x[b]

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[a] No higher probably than 1/30,000.
[b] Estimate based on sparse data.
[c] Upper 95% confidence limit is around 16-fold.

study found only 2.2% of 318 couples with abnormalities, but noted that in only a small proportion had other causes of repeated abortion been ruled out prior to cytogenetic investigation. In the study of Mennuti et al. (40), other causes of multiple abortion had been excluded prior to investigation.

Most of the chromosome abnormalities reported in couples with recurrent abortion are structural rearrangements. But there are a significant number of numerical abnormalities as well. These include, in particular, 47,XXX (and mosaic patterns) and mosaic 45,X individuals. (See, e.g., Ref. 30, 33, and 44.) The occasional rare XXX cells seen in peripheral blood may likely be simply consequences of aging, and not reflect an abnormal gonadal line (see, e.g., Ref. 40).

While the pattern appears relatively consistent with regard to the association of gross cytogenetic abnormalities and recurrent abortion, there is considerable uncertainty about the significance of normal cytogenetic variation. For example, suggestive evidence of the association of a long Y chromosome (Y/F ratio greater or equal to 1.00) with fetal loss has been reported by, among others, Patil and Lubs (45) and Nielsen (42). These two studies found a greater history of previous fetal loss in mothers of males with long Y chromosomes than in controls (33% vs 16% and 22% vs. 13%, respectively). Genest (20) in an evaluation of 51 married couples with repeated abortion found a high proportion of those with Yq+ (19.6%) compared to the expected rate, reported by others, of 1.5%. On the other hand, Verma et al. (59) found no difference between 15 such families and 15 controls. The mean in both groups was greater than 1.0 (1.046 ± 0.106 in "cases;" 1.068 ± 0.096 in controls). Verma et al. suggest that the average Y/F ratio in white, black, and East Indian populations is greater than 1.0, and that previous control values used were in error, so that a Y/F ratio > 1.0 should not necessarily be labeled "long."

In principle, methodological variation should not account for these differences among studies, at the Y/F ratio is, according to conventional interpretation, relatively constant. Nevertheless, it appears otherwise difficult to account for the differences between the above report and that of Nielsen (42) and of Patil and Lubs (45), who also had internal control groups and found in them a proportion of Y/F ratio ≥ 1.00 at a frequency of no more than about 1%. Perhaps some unrecognized difference in technique has led to the discrepancies.

The association of heteromorphisms (in addition to those involving the Y chromosome) with reproductive loss has been examined by Pantzar et al. (44) who report simply no association with any. The data of Carothers et al. (7) were also consistent with no effect.

There have not been to my knowledge studies of female infertility per se, aside from instances of multiple miscarriages and of primary amenorrhea. An extensive report of cytogenetic abnormalities associated with delayed sexual development in females has been done by Reindollar et al. (47). The data from this study are summarized in Tab. 21. It is noteworthy that it is only in hypergonadotrophic hypogonadism (i.e., ovarian failure) associated with delayed or absent menses that there is a significant association with cytogenetic abnormality.

The variation of detectable abnormality by phenotype is even more strikingly illustrated by a report from Italy by Anglani et al. (2). This was a report on 231 females aged 0 to 48 yr referred because of a suspicion of a sex chromosome abnormality (probably 45,X or 45,X/46,XX mosaics). They were subdivided into 5 different phenotypic groups, with the results presented in Tab. 22. Several things are noteworthy. Short stature is a

Tab. 21. Chromosome abnormalities in females with "pubertal" anomalies. Abstracted and calculated from Reindollar et al. (47).

I. By gonadotrophic status

Hypergonadotrophic hypogonadism	69/109 = 63%
Eugonadism	0/66
Hypogonadotrophic hypogonadism	0/77
Total:	69/252 = 27.3%

II. Type of abnormality within hypogonadotrophic subgroup

45,X	24	
45,X mosaicism (no structural abnormality)	8	
46,Xi(Xq) present	18	
Other X structural abnormality	7	
All Turner syndrome genotypes	57	(52.3%)
XO/XY and related genotypes	12	(11.0%)
XY (male pseudohermaphrodites)	6	(5.5%)
XX	34	(31.2%)
Total:	109	

well-known sign of Turner syndrome (45,X). But in females (under age 15 yr) with short stature only (height ≤ 3rd percentile for age), there was a high rate of cytogenetic abnormality (35%), including 8/57 cases with a nonmosaic 45,X pattern. This is a surprisingly high proportion. Those with physical stigmata of Turner syndrome (e.g., webbed neck, coarctation of the aorta, etc.) were presumably screened into another group, but it is possible that there was additional phenotypic selection that was not recognized over and above short stature in the group of small girls. It will be interesting to see what subsequent studies of this type reveal.

With regard to other types of abnormalities of sexual differentiation conjectural estimates concerning different categories of events based on personal observations and personal communications from J.L. Simpson appear in Ref. 29. Among male pseudohermaphrodites probably not more than 25% have a mosaic 45,X/46,XY pattern (although this depends on the effort made in seeking a minor cell line). And among true hermaphrodites about 20% have a Y chromosome (10% 46,XX/46,XY; 5% 46,XX/47,XXY; 5% 45,X/46,XY). The proportions of cytogenetic abnormalities among males with "delayed" or absent puberty, gynecomastia, and related abnormalities, have not to my knowledge been estimated, but obviously a significant proportion has a 47,XXY pattern or others associated with Klinefelter syndrome. Among males with hypospadius and other aspects of incomplete sexual development which are not extensive enough to result in classification as pseudohermaphrodites, there must be a significant proportion with a (mosaic) 45,X line.

SUMMARY AND CONCLUSIONS

Table 23 presents a summary of estimates of chromosome abnormalities associated with various categories of morbidity and mortality. As noted above this includes only the impact of abnormalities in which prezygotic (or zygotic) origin is presumed, or at least cannot be reasonably excluded. If this restriction were to be relaxed then a large proportion, if not a majority, of malignancies would also be included, as well as an uncertain proportion of disorders associated with aging and/or autoimmune processes.

Tab. 22. Chromosome abnormalities and phenotype in those with findings suggestive of Turner syndrome. Abstracted and calculated from Anglani et al. (2).

I. Complete "Turner syndrome phenotype" and chromosome anomalies by age

<1 yr	6/6 = 100%	all 45,X
1-14 yr	6/13 = 69%	6 45,X; 3 i(Xq) or del X
≥15 yr	21/26 = 80%	13 45,X; 3 i(Xq) or del X 5 45,X/46,XY or r(Y)

II. Short stature (only) in females (1-14 yr)

20/57 = 35.1% with abnormal chromosomes

8 45,X
1 45,X/46,XX
1 46,X,i(Xq)
5 45,X/46,X,i(Xq)
2 45,X/46,X,r(X)
2 46,X,del(Xp)
1 46,X,t(Xp;Yq)

III. Primary amenorrhea without short stature, or Turner syndrome phenotype in females > 15 yr

6/64 = 9.4% with abnormal chromosome (excluding 46,XY)

XY absent	0 45,X 2 45,X/47,XXX 1 45,X/46,X,i(Xq) 2 46,X del(Xq)
XY present	6 46,XY 1 45,X/46,XY

IV. Short stature and primary amenorrhea in females > 15 yr

20/36 = 55.5% with abnormal chromosomes

XY absent	9 45,X 1 45,X/46,XX 4 46,X,i(Xq)(2 45,Xmos) 1 46,X,del(Xp) 1 46,X,del(Xq)
XY present	2 45,X/46,XY 2 45,X/46,X,r(Y)

The estimates for any specific category depend upon factors associated with the prevalence rate of abnormalities in conceptuses and in livebirths. Such variables as the proportion of older women in the childbearing population and the proportion electing prenatal diagnosis and selective abortion will of course have a strong influence on these estimates and may vary significantly among populations (see footnote to Tab. 23).

Nevertheless, it is clear that aneuploidy makes a large contribution to human morbidity and mortality. It takes its toll from the earliest stages of life well through the reproductive years, and even beyond. The methods of "preventing" aneuploidy in humans today, i.e., selective abortion following amniocentesis or contraception in older women and other high risk groups, are crude and indirect. By their very nature they can "prevent" only a proportion of aneuploid offspring even in those individuals to whom these are acceptable ethical options.

Tab. 23. Estimates of cytogenetic abnormality associated with various types of morbidity and mortality. Estimates will vary with live-birth prevalence which is a function in part of the proportion of children born to older mothers and the use of prenatal diagnosis and selective abortion. These estimates (particularly those on postnatal mortality and morbidity) are on populations around 1975 in developed countries. At that time the proportion of mothers 35 yr or older was roughly 6-10%, with some variation. Prenatal diagnosis and selective abortion was not in any significant use at that time.

Types of morbidity and mortality		Estimates of cytogenetic abnormality
Early embryonic death in unrecognized pregnancies		?? 33-67%
Recognized embryonic and fetal deaths (≥ 5 weeks)		About 30% total. Rate varies from 50% at 8 to 11 weeks to about 5% in stillbirths (≥ 28 weeks)
Infant and childhood deaths		5-7%
Birth defects		4-8%
Congenital heart defects		13%
Three independent birth defects and mental retardation (all of unknown etiology)		5.5% (case rate minus control rate)
Mental retardation (excluding fragile-X)	IQ < 20	? 3-10%
	IQ 20-49	12-35%
	IQ 50-69	? 3%
Other developmental disability		? 1-3%
Criminality (defined operationally by presence in security setting)	Males in "ordinary" prisons	0.8%
	Males in settings for criminal psychopaths, retarded criminals, etc.	3.0%
	Females in prisons	0.4%
Infertility in males		2% (15% in those with azoospermia)
Male pseudohermaphrodites		≤ 25%
True hermaphrodism		20%
Multiple miscarriages in couples		2-5% (depending upon screening out of other causes)
Females with pubertal anomalies		27%
Females with hypergonadotrophic hypogonadism		65%

The theme of this Symposium--the search for underlying causes of aneuploidy and for etiological mechanisms by which it is manifested--is a difficult and challenging one. But it is a goal justified by the hopes that discoveries in this field may result eventually in primary methods of diminishing, if not abolishing, this affliction of our species.

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DISCUSSION

[Unidentified speaker]: Is the impact of aneuploidy greater in populations which have been exposed to known environmental hazards?

HOOK: Not to my knowledge. [Editor's note: For further discussion of this point, and references particularly with regard to radiation, see E.B. Hook and I.H. Porter, eds. (1977) Population Cytogenetics, Academic Press, New York.]

BOND: If you were trying to explain the high prevalence of aneuploidy in human populations, then you might be forced to conclude that aneuploidy has a selective advantage in man, or else it is selectively neutral. Is there any evidence for this?

HOOK: There is no direct evidence. But one speculation is that aneuploidy is so prevalent so that human pregnancies can be spaced appropriately. Perhaps there is some optimal time from one pregnancy to another. Before people could regulate fertility, natural processes which interspersed a few abortions between pregnancies may have given the preceding child a better chance to survive. But now that we can control fertility, such a putative, protective effect is no longer necessary. A consequence of that argument is that there is something about pregnancies right after the successful birth of an euploid child that may be associated with more frequent aneuploidy. But, to my knowledge, there are no data on this.

DOWN SYNDROME: ECONOMIC BURDENS AND BENEFITS OF PREVENTION

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INTRODUCTION

Not too many years ago, the principal methods of preventing aneuploidy were harsh and by no means certain. Persons with aneuploidy could be sterilized or confined in institutions. Other high-risk persons could be counseled not to have children. In some cases, lifesaving medical care was withheld from newborn handicapped children. In recent years, however, with the development of the amniocentesis procedure to detect aneuploidy and other genetic defects, prevention has become both relatively certain and financially inexpensive, raising obvious economic issues of how much to invest in preventing aneuploidy.

ECONOMIC ISSUES

Clinicians and researchers in the field of aneuploidy generally have little interest in the results or methods of economic analyses per se. For the most part, they are dedicated persons who are already convinced of the importance and value of their efforts. What they want economists to develop are dollar figures on costs and benefits that they can use to convince Congress, State legislatures, hard-nosed budget analysts, and government agencies that more resources should be invested in their research activities and operating programs. If economic analyses do not confirm the desirability of expanding these activities, I suspect that most of these persons will dismiss these analyses as being flawed.

Economists, however, take themselves more seriously. Two basic questions usually underlie the analyses they undertake. First, should increased resources be allocated to a particular area, e.g., research on methods of averting aneuploidy or expansion of clinical programs to prevent aneuploidy? Second, how should resources devoted to aneuploidy be distributed to achieve the greatest social benefits, e.g., in what areas should research on aneuploidy be expanded? These two questions are sometimes referred to as the "how much" and the "how to" issues.

In order to help answer these questions, economists make use of the following types of analyses:

(a) Cost-of-condition analyses. Many estimates have been made of the costs of various conditions, e.g., mental illness, mental retar-

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dation, alcoholism, road accidents, etc. In cost-of-condition analyses, we seek to measure the extent to which social welfare (well-being, happiness) is reduced as a result of a part of the population having a particular condition, e.g., aneuploidy. The primary purpose of these analyses is to estimate the seriousness of the effects on society of a particular condition. Sometimes the estimated size of the cost of a condition is used to demonstrates the need to provide treatment and/or to find ways of improving methods of treatment for a particular condition. However, by themselves, cost-of-condition analyses cannot justify increasing the resources used for treatment or research programs. These decisions require cost-benefit analyses.

(b) Cost-benefit analyses. In cost-benefit analyses we seek to compare the costs of resources used for treatment or research programs with the increase in social welfare (the benefits) that occur. Cost-benefit analyses are used both to determine if the resources devoted to a particular area should be increased (or decreased), i.e., the "how much" issue, and to compare different programs to determine which ones should be expanded, i.e., the "how to" issue.

(c) Cost-effectiveness analyses. Cost-effectiveness analyses are almost always less difficult to perform than cost-benefit analyses. In these analyses the costs of different methods of achieving a specific outcome are compared. If, for example, it were decided that the incidence of aneuploidy should be reduced by 5%, then the analytical task would be to identify which program, or combination of programs, would accomplish this goal at the lowest cost. Should women at high risk be counseled not to have children? Should expanded use of amniocentesis be undertaken, and if so, on what groups should it be targeted? By its very nature cost-effectiveness analyses are used to choose between alternative ways of using resources, i.e., the "how to" issue, and provide little guidance in determining "how much" to spend.

A variant of cost-effectiveness analyses occurs when costs are predetermined and analysts seek to identify the program or programs that will provide the greatest output for the expenditure. However, such analyses invariably involve a comparison of both costs and benefits of various spending opportunities and are more appropriately subsumed under our second category of cost-benefit analyses.

Four observations will emphasize the importance of these analytic methods. First, in principle, cost-benefit analyses should be conducted only for that part of a program which is being considered for expansion or contraction. Decisions are usually made on the basis of expanding programs by some percentage amount, e.g., 10%, and it is only the costs and benefits associated with this increased expenditure that should be considered in making a decision to make this expenditure. In principle, additional resources should be allocated to a program as long as the increase in benefits that results from expanding the program (marginal benefits) exceeds the increase in costs (marginal costs).

Second, as programs expand, the marginal cost-benefit ratio (i.e., benefits divided by costs) should decline. For example, a program to prevent aneuploidy would probably initially focus on the groups of individuals with the greatest risk of having a child with aneuploidy, e.g., pregnant women over age 40 yr or women who had previously given birth to a child with aneuploidy. As the program expanded, the number of cases found per dollar of expenditure would decline as the program served women who were increasingly less likely to give birth to a child with aneuploidy.

Third, regardless of whether or not economic analyses are conducted, decisions concerning the amount of, and allocation of, resources to research and clinical programs in aneuploidy must be made. One reason is that budget constraints inevitably preclude funding all programs and research requests. A second reason is that often only one of a number of competing alternatives can be carried out at a time. For example, training a handicapped person for one type of job obviously precludes training him or her for another type of job at that point in time.

Finally, although resource allocation decisions for programs in aneuploidy, as well as for most other programs, are based on estimates of costs and benefits, these estimates are often intuitive, being based on perceptions and hunches rather than on a rigorous assessment of existing data. Although they cannot eliminate all judgement in resource allocation decisions, economic studies may provide substantial assistance to policymakers who must decide whether to increase (or decrease) resources going into a given area, and who must also decide which of the competing programs and research proposals should be funded or given an increase in funding.

Economists invariably begin studies in areas such as aneuploidy by asking basic questions about data that are needed to prepare their estimates, such as: How often does aneuploidy occur? What are the costs of different methods of preventing a case from arising? What is the likelihood that a person with aneuploidy will be gainfully employed? What are the costs of caring for persons with aneuploidy? The answers that are given to these questions are usually subject to wide error, which in turn sets the limits as to the accuracy and reliability of subsequent estimates of the social cost of aneuploidy or to the costs and benefits of treatment. Economists have no secret tricks that can improve the quality of existing data.

In this chapter I will develop a model for conducting economic analyses of aneuploidy and present some empirical estimates of the costs of aneuploidy and the benefits of prevention. Persons who disagree with the empirical data I use may utilize their own estimates of essential variables to determine how the conclusions are affected.

THE MODEL

The Effects of Aneuploidy

Aneuploidy is one of a large number of diseases, accidents, and genetic conditions which adversely affect people's well-being. Sometimes we will use a broader term, such as "genetic condition" or "disability." In these cases it should be understood that the concepts being discussed are as applicable to aneuploidy as to other handicapping conditions. Following Nagi (11), the ways in which chronic conditions affect people can be categorized as follows.

Impairments. The immediate effect of a permanent pathology, such as aneuploidy, is to create an impairment. An impairment is defined as "... a psychological, anatomical, or mental loss, or other abnormality, or both" (11). Examples of impairments are loss of limb, loss of use or restricted use of limb, diminished mental capacity (including mental retardation and mental illness), eye impairment, ear impairment, reduction in motor control of body, heart impairment, and disfigurement.

Handicaps. Impairments, in turn, lead to "... limitations in function at higher levels of activity" (11). These functional limitations are usually referred to as handicaps. "Handicaps or functional limitations can be of a physical, emotional, mental, or sensory nature" (Ref. 11, p. 3). Examples of handicaps are: (a) sensory limitations, such as limitations in sight, hearing, taste, etc.; (b) physical limitations, such as limitations in walking, climbing, lifting, bending, and reaching; (c) mental limitations, such as reduced capacity to reason, or reduced capacity to functionally comprehend the world (e.g., paranoia, depression); and (d) other limitations, such as reduced bodily vigor and durability due to organic problems (e.g., heart problems).

In effect, handicaps are the direct objective affects on the functioning of the individual due to conditions such as aneuploidy. The dividing line between impairments and handicaps becomes blurred in some instances, e.g., mental retardation and reduced capacity to reason.

Premature death. In some cases, the length of life is reduced because of a chronic condition. This is a major consequence of aneuploidy.

Ultimate effects. Handicaps, in turn, may affect the way an individual responds to social expectations and roles, and the way society responds to him or her. I use the term "ultimate effects" because no other existing term encompasses the full range of effects that may result from conditions such as aneuploidy. By ultimate effects I mean all of those consequences of genetic abnormalities or diseases, or injuries that affect people's happiness or well-being, or the happiness and well-being of their family and friends and the rest of society. Among the more important of these ultimate effects are:

(a) Loss of, or reduction in, work capacity leading to a reduction in the production of goods and services (usually referred to as work disability). This, of course, leads to a reduction in earnings.

(b) Loss of, or reduction in, ability to carry on productive activity for which pay is not normally received, such as housework, child care, family budgeting, house and lawn maintenance, and other normal activities. Volunteer work should also be included here. Although these activities do not generate a flow of cash income, they contribute to individual and social well-being. In fact, in many cases, individuals must judge that these nonwork activities make a greater contribution to their well-being than would the same amount of time in paid work.

(c) Loss of, or reduction in, ability to perform nonwork activities (consumption and activities related to consumption) normal for a person's age and sex such as visiting friends and relatives, shopping, eating in restaurants, attending movies, driving a car, engaging in sports, enjoying art, sightseeing, going from place to place, and all of the activities that people normally do.

(d) Reduced likelihood of normal personal relationships, including fewer friends and less, or no, chance of marriage and children.

(e) Psychic distress such as depression, frustration, anger, fear of death, feeling of being cheated in life, etc.

(f) Presence of pain.

The above effects refer to the ways in which handicaps directly affect individuals with chronic conditions. These handicaps may also affect the relatives and friends of handicapped individuals. A family member may forego paid work in order to provide care, or family stability may be threatened, possibly leading to a divorce.

In addition to directly affecting the way people conduct their lives, handicaps often create substantial service needs. Among services that are likely to be needed for persons with aneuploidy are: (a) special medical treatment to ameliorate, cure, or prevent the effects of the condition; (b) special education services; and (c) other supportive services such as counseling, vocational services, and day care. Services are usually provided directly to the handicapped person, but may also be extended to relatives or friends, e.g., counseling. Services may also be provided to parents or prospective parents in order to prevent the occurrence of the handicapping condition, or to society as part of prevention and treatment efforts.

Although not exhaustive, these represent a listing of the more important and better known of the ultimate effects of chronic conditions. Two further observations are made. (a) Premature death affects social well-being in ways comparable to those listed for morbidity. In addition, of course, most people place a great value on continued life, above and beyond any other losses incurred. (b) The number of ultimate effects becomes enormous as the effects of handicapping conditions ripple through society over time. We have listed only some of the more important and obvious of these effects. We can identify many other examples. Families may decide to reduce the number of children they have. Social savings and capital formation will be reduced as a consequence of reduced earnings and the use of resources to provide services to handicapped individuals. Siblings may forego college with resultant effects on their lives and the lives of their children.

ECONOMIC CONCEPTS

We can now more rigorously define the economic concepts described above. The social cost of aneuploidy is equal to the total of the values of all of the ultimate effects caused by the condition. This total includes the value of lost output (both paid and unpaid) by the person with aneuploidy as well as his or her relatives, the value of loss of nonwork activities, the value of psychic distress, the value of all of the prevention and treatment programs, the value of research activities into aneuploidy, the value of all of the losses associated with premature death caused by aneuploidy, and many others as the effects of aneuploidy ripple through society. The total can be represented as:

$$S = e1 + e2 + n1 + n2 + n3 + p1 + p2 + c1 + c2 + c3 + \ldots$$

where e's represent losses of earnings and other unpaid work, n's represent the values of losses of nonwork activities, p's represent the values of psychic losses, and c's represent the costs of various treatment and prevention programs. A comprehensive listing of all of the components of social cost would probably fill this page.

Measures of social cost can take two forms. First, one can measure the social cost incurred during a single time period, usually a yr, for all persons with aneuploidy. Second, for newborn persons with aneuploidy, we can estimate the social cost over their lifetime.

In general, we assume that the values associated with the various effects are zero or negative. Occasionally, optimists describe a positive value, as when families are bound together more closely by the common task of caring for a handicapped child.

Many variables affect social costs. If persons with aneuploidy are assisted to return to work, or to leave institutions and live in the community, social costs will decline. If standards of care rise, social costs may rise.

Cost-benefit analysis is directly related to social costs. Suppose a program is expanded or a new program created to prevent aneuploidy or to provide care to persons with aneuploidy. The value of these additional resources and other costs directly related to the program, such as the costs of traveling to and from the place of treatment, represent the cost side of the cost-benefit comparison. These costs also represent additions to the social cost of aneuploidy and will be reflected in increases in some of the components in the social cost equation.

Hopefully, however, the program will reduce other losses associated with aneuploidy causing a decline in other terms in the social cost equation. These reductions in other variables in the social cost equation represent the benefits of the program. Obviously, when benefits exceed costs, the program is worthwhile.

There are several different ways in which these comparisons of costs and benefits can be made, as follows: (a) benefits can be divided by costs giving the benefits per dollar of expenditure for the entire period in which benefits accrue; (b) the average annual benefits can be calculated giving an annual return (comparable to the rate of return on business investment); (c) the payback period can be calculated showing how much time is required before costs are fully recovered through benefits; (d) the total net benefits can be calculated by subtracting costs from benefits; and (e) costs and benefits can be set forth in a systematic manner in order to assist the decision-maker to arrive at a judgement. The advantages and disadvantages of the first four of these techniques are discussed elsewhere (4). The fifth approach, simply setting forth costs and benefits in a systematic manner, has much to recommend it in view of the virtual impossibility of expressing many important benefits in monetary terms (which is necessary if they are to be added together).

In cost-effectiveness analysis we identify the least costly way of achieving a predetermined goal. It is not necessary that this goal be expressed in terms of one of the ultimate effects of aneuploidy. One could, for example, set a goal of reducing aneuploidy by a given number of cases.

Problems in Conducting Economic Studies

There are many empirical and conceptual problems in calculating and interpreting these economic concepts.

Problems of missing data. Cost and benefit calculations can be no better than the data on which they are based. Unfortunately, much needed data are of poor quality or do not exist. For example, we have little reliable data on how many persons with Down syndrome survive to different ages. We do not know how many are employed as adults, or what they earn. We know little about the services they receive, or how much these services cost.

Problems of variables without a market (monetary) value. Many of the ultimate effects of aneuploidy do not have an easily determined market value. This is obviously true for psychic losses and for losses of most consumption activities, such as playing sports and shopping.

Problems of different weighting of components of costs and benefits. Different people will come to very different conclusions from cost-benefit analyses because they do not place the same value on different types of costs and benefits. Economists are invariably concerned about future losses of earnings. Prospective parents, however, will pay little attention to such esoteric economic concepts in deciding whether or not to prevent (or to attempt to prevent) the birth of a child with aneuploidy. One means of preventing aneuploidy is by abortion of an affected fetus. Almost all people place a negative value on abortion. In some cases, prospective mothers are so horrified at the prospect that they prefer to give birth to a handicapped child, future earnings losses and treatment expense being of much less importance than the emotional costs (or negative benefits) of abortion. In some cases, people seek to prevent any abortions from occurring even though the abortions occur to unrelated individuals. To them, the humanitarian and moral costs of abortion outweigh any financial benefits of preventing aneuploidy. In cost-benefit analyses the negative values associated with abortion could be treated as an addition to costs, but are more correctly regarded as a negative benefit. The conclusion is the same in either case.

Problems of joint benefits. Programs to prevent aneuploidy also generate additional benefits through prevention of other serious chronic conditions. Genetic counseling is not limited to advising clients about the likelihood of having a child with aneuploidy. Amniocentesis can detect many fetal problems other than aneuploidy. Technically, cost-benefit analyses should encompass all benefits attributable to these prevention programs, regardless of whether the benefits are from preventing aneuploidy or from preventing some other serious condition.

Problems of distribution. The costs of preventing aneuploidy, or subsequently providing care to persons with aneuploidy, must often be paid by taxpayers, while the benefits of prevention accrue to prospective parents and the benefits of treatment programs accrue to persons with aneuploidy and their relatives. Technically, it cannot be argued that these benefits justify costs, even if benefits are much greater than costs, if the individuals bearing the costs are different from the individuals receiving the benefits. Judgements that programs should be undertaken that result in taking resources from one group in order to benefit another group must be made through a political process. Economic analyses can unambiguously support the expansion of programs only when the gains to taxpayers exceed the costs they bear, e.g., if the taxes paid to support prevention programs are less than would otherwise be levied to provide residential care and other services to persons with aneuploidy.

Problems of defining the appropriate decision units. Despite the insistence of economists on the importance of conducting cost-benefit calculations based on marginal changes in programs, the fact is that it is extremely difficult to identify the costs and benefits associated with changes in program size. It is, in many cases, difficult or impossible to determine who would be the additional persons served if a program were to expand, and to ascertain what would be the benefits associated with these additional individuals.

Problems of discounting. Most of the benefits, and sometimes the costs, of programs in aneuploidy take place in future years. However, future dollars earned or expended on residential care are worth less today than their nominal future amount. For example, how much would you offer for $100 payable in 1 yr? If you were currently able to obtain a 10% return on your savings, then the most that you should offer would be $90.91 since in one yr, this amount will draw $9.09 in interest and be worth $100.

The calculation of present value (PV) of a future cash amount is equal to:

$$PV = A(n)/(1 + r)^n$$

where PV = present value, A(n) = amount paid n years in the future, and r = rate of discount (or interest). If there is a string of future dollar amounts (e.g., of earnings or residential care payments), the present value of the sum of these future amounts will be calculated by the formula:

$$PV = a(1)/(1+r) + a(2)/(1+r)^2 + \ldots\ldots\ldots + a(n)/(1+r)^n$$

Obviously, the higher the rate of interest, the lower the present value. For example, the present value of $100 in 1 yr discounted at 20% is $83.37, while discounted at 5%, it is $95.24. Moreover, the greater the number of yr before the dollar amount is earned or paid, the lower its present value. Discounted at 10%, $100 payable in 5 yr will be worth $62.09; in 10 yr, $38.55; in 20 yr, $14.86; and in 40 yr, $2.21.

This creates a crucial problem in evaluating programs to prevent aneuploidy. Although costs are incurred immediately, savings on residential care costs and increased earnings may not begin for 20 yr or more, so that the choice of discount rate will greatly influence the size of the present value of these benefits of prevention. However, there is uncertainty as to which discount rate is most appropriate since there are many different rates of return depending upon the type of investment. Some analysts use 2 rates and argue that this provides upper and lower bounds to measures of benefits and costs. However, this greatly increases the number of tabulations and gives frustrated decisionmakers 2 sets of improbable data. In consequence, I prefer to use a single intermediate rate. I use a discount rate of 7% in subsequent calculations. Although 7% is considerably lower than current interest rates, it must be remembered that current rates are greatly influenced by the expectation of inflation as well as current monetary policy. I believe that 7% represents a reasonable estimate of the long-term interest rate.

Technical cost problems. Economists define the economic (or social) cost of prevention or treatment programs for conditions such as Down syndrome as the additional use of resources that must be incurred in operating these programs. Similarly, the social cost of a handicapping condition encompasses only resources that are used for purposes that they would not be used for in the absence of the condition. One cannot always arrive at these totals by adding together the expenditures of one or more programs. Some of the more important adjustments that must be made to program expenditures to arrive at economic or social costs are the following.

(a) Resources expended to provide room and board (hotel costs), normal medical care, and other normal activities of life would be used for these purposes in the absence of the handicap and should not, therefore, be included in measures of the social costs of aneuploidy or the costs of programs to prevent aneuploidy or care for persons with aneuploidy.* In consequence, Social Security and Supplemental Security Income (SSI) payments to disabled persons are not counted as social costs, nor are Medicare or Medicaid payments for normal medical care. Although these payments represent costs to taxpayers, they are benefits to recipients and such payments are considered transfer payments from one part of society to another. The only part of the expenditures of transfer-payments programs that should be counted as social costs is the costs of administering these programs. Similarly, when estimating the social cost of institutional or other residential care programs, that part of the expenditures of these programs used to provide normal living expenses should be subtracted from program costs to determine economic costs. It is worth observing that failure to make this adjustment for normal living costs may result in double-counting. For example, suppose estimates are made of earnings loss due to aneuploidy as well as the cost of institutional care unadjusted for normal living expenses. The earnings loss encompasses the funds that would be used for normal living expenses in the absence of aneuploidy. In effect, normal living expenses would be included in both the estimate of lost income and the costs of residential care.

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* Of course, the amounts expended for these purposes might differ between handicapped and nonhandicapped persons, but this is a technical consideration for which there is no meaningful adjustment.

(b) In calculating educational costs due to aneuploidy, only that amount expended in excess of regular education costs should be counted since these normal education costs would be incurred in the absence of aneuploidy.

(c) In some cases (e.g., residential care), buildings and equipment are used that are publicly owned and do not enter in the budget. However, the fair rental value of these capital items represents a social cost that should be included in measures of cost.

(d) If volunteer help is provided to programs, a value should be imputed to these services and added to measures of social costs.

(e) If persons receiving treatment lose earnings because of time lost from work or incur travel costs during treatment, these costs should be added to estimates of economic costs.

Technical problems of benefits. Several technical problems arise in the measurement of social benefits.

(a) Successful prevention or rehabilitation may increase future earnings and the payment of taxes, and, in addition, reduce transfer payments for income support and normal medical care. However, only the increase in earnings should be counted as a benefit. Increases in taxes and reductions in transfer payments serve the purpose of distributing part of the increase in earnings to taxpayers. It would cause double counting of benefits if increases in taxes and/or reductions in transfer payments were added to increases in earnings in order to measure benefits.

(b) Future earnings will be influenced by economic growth and inflation. For purposes of calculating the benefits due to treatment or prevention programs, future increases in earnings due to economic growth should be counted as a benefit if they would not otherwise have been received. However, future increases due to inflation are not a benefit. Usually, future earnings are expressed in terms of the price levels prevailing in a given year.

(c) Future and present earnings may be affected by unemployment in the economy. Should loss of earnings among persons with aneuploidy be counted as part of the social cost of aneuploidy if the persons are unemployed, not because of inability to work, but because of economic conditions? Similarly, is it worthwhile to provide vocational services to disabled persons if economic conditions make employment unlikely. In answering these questions, it must be observed that there are several different major reasons for unemployment. Some unemployment is inevitable and even beneficial. At any point in time, some workers will be losing their jobs because some companies fail, new technologies make their jobs obsolete, or changes in consumer tastes reduce the need for providing some types of goods. In addition, some workers are newly entering the work force, and some are returning to work after a spell of illness. It usually will require a short period of time before these temporarily (hopefully) idle workers locate employment; unemployment during this job search period is defined as "frictional unemployment." It should be assumed that frictional unemployment will exist when calculating the future earnings of persons, and future earnings reduced accordingly.

But suppose persons with aneuploidy are unable to locate work, not because of their handicaps, but because of depressed economic conditions. This type of unemployment is usually described as "cyclical" unemployment. Should loss of earnings due to this type of unemployment be considered part of the social cost of Down syndrome? Should the benefits of rehabilitation programs be reduced? In my judgement, the answer to these questions is negative. "Cyclical" unemployment represents a breakdown of the economy and the failure of government to maintain reasonably full employment. It is absurd to add part of the costs of the recession to the burdens already borne by an already highly disadvantaged group in our society. It would be equally absurd to compel disabled persons to wait until other persons were hired before being considered for employment. It should also be noted that recessions are short-term problems that should be countered with appropriate monetary and fiscal policies. The problems caused by aneuploidy are lifelong, and should be countered with appropriate treatment and prevention programs without regard to temporary malfunction of the economy.

Ambiguity of benefits of preventing aneuploidy. In most instances, the benefits of preventing a disabling condition are clear, at least at a conceptual level. Normally, one compares how well off the individual and society would be in the absence of disability with how well off the same individual and society would be if the person were disabled. In the case of aneuploidy, however, prevention takes place by preventing the birth of a person with aneuploidy. High-risk persons may choose not to have children, parents may choose to have children while younger and at less risk of having a child with aneuploidy, or pregnant women may choose to undergo amniocentesis and abort if the child has aneuploidy (or some other disabling or life-threatening condition).

This leads to two possibilities. In families that predetermine the number of children that they will have, preventing (or reducing the likelihood of) aneuploidy means that they replace a child with aneuploidy with a child that (hopefully) does not have this condition. Benefits are measured by comparing the level of social welfare in the case of a child with aneuploidy and the case of a child without aneuploidy using such variables as differences in earnings, costs of care, and costs of education. The problem is that we are making life and death decisions on the basis of comparisons of social welfare for two different potential individuals, a procedure that is acceptable only if we place little or no value on the life of an unborn (although conceived) child.

The second possible consequence of prevention is that a family will have fewer children than they otherwise would have had. Although I have previously described this as the nonreplacement case, the term is misleading since the number of children may be reduced by 1 or more. How can one calculate the social losses of having fewer children? And how does one measure benefits when there is no "normal" child against whom comparisons can be made? Actually, the only feasible approach in this nonreplacement case is to estimate benefits only for persons actually born, in other words, to ignore the costs and benefits that would have been incurred or enjoyed by persons who were not born because of prevention efforts, assuming they had been born. This assumption has a major effect on the way in which benefits are calculated for this type of prevention. In particular: (a) There are no future earnings, unpaid work, or consumption activities to count as benefits. Earnings and other productive activities that might have been undertaken by the persons who were not born are not considered. (b) The benefits from prevention in the nonreplacement case are the costs that are not borne by society for their care and support. These include institutional and special education costs. Unlike the "replacement case," normal consumption and normal medical care are not subtracted from these costs if they will be borne by taxpayers. In addition, any anticipated reductions in income and medical support payments (Social Security, SSI, Medicare, Medicaid, etc.) are counted as benefits in the nonreplacement case, although these payments are regarded as transfer payments in the "replacement case."*

Another possibility of prevention efforts is that some people will increase the number of children they have because of the reduction in the fear of having a defective infant, presumably adding to the benefits of prevention programs. This is an example of the complex ways in which the effects of prevention ripple through society. Usually, these important effects of prevention programs are overlooked in evaluation studies. However, I feel no need to add an "enhancement case" to the "replacement" and "nonreplacement" cases that have already been established.

Conclusions. Clearly, economic analyses, no matter how well-crafted, can neither fully measure costs and benefits, nor resolve conflicts in the ways in which people value various components of costs and benefits, nor fully resolve other conceptual problems. One should not conclude, however, that such analyses have little value. To the contrary, analyses that identify and refine cost and benefit information are of enormous utility to decision-makers who are called upon to determine when to expand and when to contract programs. It is essential, however, that it be recognized that while these analyses assist decisionmaking, they do not substitute for the individual judgements that must be made or the political processes that must be invoked to resolve differences.

SOCIAL COST OF DOWN SYNDROME

In this section, we will develop an estimate of the reduction in social well-being that results because a part of society has Down syndrome. The great majority of persons with aneuploidy have this condition.

Prevalence

The first step is to determine how many people with Down syndrome are living in various age groups, and how many people with this condition would be alive if they had normal mortality.

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* Families that continue to have children after an initial termination of pregnancy may still fall in the nonreplacement category as long as they have fewer children than they would otherwise have had.

There have been numerous studies of the incidence of Down syndrome. In general, an incidence rate of approximately 1 in 700 for populations of European origin appears to emerge from these studies (13). Apparently, the incidence is somewhat lower among nonwhite persons and will be affected by changes in the age at which women choose to have children, as well as the utilization of elective abortion (9). In recent years, the incidence of Down syndrome has apparently declined as a consequence of the growth of prevention efforts. We should note that many aneuploid fetuses die before or during birth from natural causes. Although these deaths undoubtedly create economic costs, we will not consider this cost element in subsequent calculations.

Mortality among persons with Down syndrome is much higher than for the general population, in large part because of associated physical disorders such as heart disease. Hagard and Carter (6) provide estimates of the proportion of persons who were born with Down syndrome who survived to various ages (Tab. 1, Column 2). If we assume (temporarily) that there is no mortality in the general population, and if we assume that the incidence of livebirths with Down syndrome is 1 in 700, then the percentage of living persons with Down syndrome in each age group in the population will be equal to 1/700 times the estimated survival rate of persons with Down syndrome to different ages (Tab. 1, Column 3).

Column 4 (Tab. 1) provides estimates of the percentage of persons in the general population expected to survive to specified ages (17). Dividing Column 3 by Column 4 provides an estimate of the prevalence of Down syndrome by various age groups (Tab. 1, Column 5).

Column 2 in Tab. 2 shows the number of persons in the United States population by selected age groups during 1983, including persons in institutions and armed forces personnel overseas (15). The number of persons with Down syndrome in each age group is derived by multiplying Column 5 in Tab. 1 by Column 2 in Tab. 2. After the age of 5 yr, population estimates

Tab. 1. Estimated percentage of persons with Down syndrome, by age.

Age (yr)	Percentage of persons with Down syndrome surviving to selected ages[a]	Column 2 X 1/700	Percentage of persons in general population surviving to selected ages, 1979[b]	Percentage of persons with DS (Cols. 3x4)
0	100%	.1428571%	100.000%	.1428571%
1	76	.1085714	98.865	.1098178
2	73	.1042857	98.597	.1057696
3	71	.1014285	98.529	.1029427
4	69	.0985714	98.474	.1000989
5	69	.0985714	98.429	.1001446
10	65	.0928571	98.271	.0944908
15	60	.0857142	98.111	.0873645
20	55	.0785714	97.613	.0804927
25	49	.0700000	96.957	.0721969
30	44	.0628571	96.303	.0652701
35	39	.0557142	95.629	.0582607
40	33	.0471428	94.749	.0497554
45	27	.0385714	93.416	.0412899
50	22	.0314285	91.323	.0344146
60	11	.0157142	83.405	.0188408
70	0	0	67.834	0

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[a] Data from Hagard and Carter (6).
[b] U.S. Department of Health and Human Services, Public Health Service.

Tab. 2. Estimated number of persons with Down syndrome. A further breakdown of the age groups between 50 and 69 yr is presented at the bottom to facilitate subsequent calculations (see text). This breakdown was obtained by assuming that the percentage of persons with Down syndrome in the different age groups was proportional to the distribution of these age groups in the general population.

Age (yr)	Number of persons in general population (in 000's)[a]	Estimated number of persons with Down syndrome	Estimated number of persons with Down syndrome who died prematurely
0	3,660	5,229	—
1	3,643	4,001	1,203
2	3,590	3,797	1,332
3	3,567	3,672	1,424
4	3,366	3,369	1,440
5	3,235	3,240	1,381
6-9	12,745	12,403	5,804
10-14	17,781	16,168	9,233
15-19	19,210	16,123	11,320
20-24	21,925	16,739	14,582
25-29	21,219	14,585	15,728
30-34	19,115	11,806	15,501
35-39	16,304	8,805	14,486
40-44	13,188	6,004	12,836
45-49	11,189	4,235	11,749
50-59	22,627	6,025	26,299
60-69	19,757	1,861	26,363
70+	18,371	—	26,244
50-54	11,153	2,970	12,963
55-59	11,474	3,055	13,336
60-64	10,745	1,012	14,338
65-69	9,012	849	12,025

[a] U.S. Department of Commerce, Bureau of the Census.

are given in 5- and 10-yr age intervals. An estimate of the average prevalence of Down syndrome within these intervals was derived by taking the average of the two prevalence levels closest to the interval. For example, for the interval of persons aged 10 to 14 yr, the average prevalence of Down syndrome was calculated by taking the average of the prevalence of Down syndrome (according to Hagard and Carter [6]) at age 10 yr and at age 15 yr. A further breakdown of the age groups between 50 and 69 yr is presented at the bottom of the table to facilitate subsequent calculations. This breakdown was obtained by assuming that the percentage of persons with Down syndrome in the different age groups was proportional to the distribution of these age groups in the general population.

An estimate of the number of people in each age group who had died but who would be alive in 1985 if they had not had Down syndrome was derived by multiplying the total number of people in each age group by 1/700 to estimate the number who would be alive if persons with aneuploidy had normal mortality rates, and then by subtracting the number of living persons estimated to have aneuploidy (Tab. 2, Column 4). Roughly, we estimate that there are 140,000 persons with Down syndrome in the United States of all ages, and about 170,000 persons who died who would have been alive had they not had Down syndrome.

Loss of Output

The next step is to estimate the reduction in Gross National Product resulting from Down syndrome. In order to determine the loss of output by persons with Down syndrome, we must estimate their current contribution to output. The first step in making this estimate is to estimate the number of persons with Down syndrome who live in institutions, and the number who reside in the community.

There is little information on the number of persons with Down syndrome in institutional care. There have been "guesses" that this percentage may be as high as 15%. Preliminary data from New York State, however, indicate that about 900 residents in institutions (the preferred term is developmental centers) in that State have Down syndrome, which comes to about 8% of the residents in New York institutions (Dr. Robert Lubin, New York State Office of Mental Retardation, pers. comm.). We will assume that about 10% of persons with mental retardation in institutions throughout the United States have Down syndrome, which adjusts the New York data slightly upward to reflect the higher "guesses" that have been made.

As of June 30, 1982, there were about 176,000 mentally retarded persons in group residences of 16 or more persons or in nursing homes in the United States (7). Of these, about 25% were age 21 yr or under, 5% were age 63 yr or over, and 70% were age 22 to 62 yr. From these data, we estimate that there are about 17,600 persons with Down syndrome in institutional care, of whom about 4,400 are age 21 yr or younger, 900 are age 62 yr or over, and 12,300 are between the ages of 21 and 63 yr.

Since we estimate that there are about 69,000 persons with Down syndrome between the ages of 20 and 65 yr in the United States, it appears that approximately 57,000 of these individuals live in small group homes or with relatives. In addition, there were about 130,000 persons in this age range who would have been living and contributing to output if they had not had Down syndrome.

Productivity loss is measured by the difference between what persons with Down syndrome actually produce and what they would have produced if it were not for their condition. In the case of persons in institutions, current productivity is assumed to be zero, as is obviously also the case for deceased persons. But what of the productivity of adults with Down syndrome living in the community? In general, we can assume that their productivity will be quite low. The majority of persons with Down syndrome will almost certainly be placed in day care centers or work activity centers with inconsequential earnings. Few will be employed on regular jobs at substantial earnings (5).

Table 3, part A, shows the distribution of the population in 1983 by age and sex, the percentage of persons with income in each age group, and the mean income of these persons (16). Part B (Tab. 3) shows the arithmetic average for men and women of the percentage with income in each age category, and of mean income. Subsequent calculations will not distinguish between male and female persons with Down syndrome as the data are too limited to justify the assumptions that would be required to create a sex-specific distribution of persons with Down syndrome.

One way of measuring the value of what people add to output is by the income they receive. Usually, economists consider only the salaries and earnings components of income on the grounds that these represent the assessments of employers of what workers add to output. Total income, as reported in Tab. 3, however, includes rental income, interest income, dividend income, and proprietor's income. Unlike some economists, however, I

Tab. 3. Estimated earnings loss among persons with Down syndrome, 1983.

A[a]

Age (yr)	Male: Number in population (000's)	Male: Percentage with income	Male: Mean income ($)	Female: Number in population (000's)	Female: Percentage with income	Female: Mean income ($)
15-19	9,308	67.17	2,604	9,198	63.47	2,271
20-24	10,377	92.62	8,966	10,619	87.96	6,641
25-34	19,876	97.10	18,230	20,298	90.76	10,021
35-44	14,713	98.27	25,234	15,344	91.18	10,871
45-54	10,746	98.00	26,222	11,493	89.84	10,307
55-64	10,266	98.19	22,765	11,767	90.53	9,033
65+	10,749	99.29	13,158	15,542	98.22	7,951

B[b]

Age (yr)	Percentage of population with income (male and female)	Average income of persons with income ($)	Number of persons with Down syndrome: Living	Number of persons with Down syndrome: Prematurely deceased
15-19	65.32	2,431	16,123	11,320
20-24	90.29	7,803	16,739	14,582
25-34	93.93	14,125	26,391	31,229
35-44	94.72	18,052	14,809	27,322
45-54	93.92	18,264	7,205	24,712
55-64	94.36	15,899	4,067	27,674

C

Age (yr)	Income loss among persons with Down syndrome ($): Living	Income loss among persons with Down syndrome ($): Prematurely deceased
15-19	25,602,000	17,975,000
20-24	117,932,000	102,735,000
25-34	350,146,000	414,334,000
35-44	253,217,000	467,175,000
45-54	123,591,000	423,898,000
55-64	61,014,000	415,174,000
Total:	$931,502,000	$1,841,291,000
Reduced by 5% to allow for proportion of income received from disability transfer payments:	$884,927,000	$1,749,226,000

Further reduced by 5% of the income loss among living persons with Down syndrome to allow for current earnings: $840,681,000

Total estimated earnings loss: $2,634,153,000 believe that Down syndrome and other handicapping conditions that inhibit work also create losses in addition to those reflected in salaries and earnings. After all, if people did not have Down syndrome, they would presumably have saved, invested, and established businesses, as other people have, and would receive various types of income in addition to earnings. Rental income, dividend income, interest income, and profits reflect components of total output as much as earnings.

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[a] U.S. Department of Commerce, Bureau of the Census, Estimates of the Population of the United States.

[b] U.S. Department of Commerce, Bureau of the Census, Income and Poverty Status of Families and Persons.

However, a problem with using income data as a measure of what persons with Down syndrome would contribute to output if it were not for their condition arises because these income data also include transfer payments from Social Security and the Supplemental Security Income programs which clearly do not reflect contributions to output. During 1982, almost 14% of the nation's personal income was derived from government transfer payments (14). Of this amount, however, about 70% was used to finance retirement programs and was not received by persons who are disabled.

The following procedures were followed to estimate current output losses due to Down syndrome. The percentage of persons in each age group in the general population with income was multiplied by the number of persons in each age group with Down syndrome, both living and deceased, including persons living in institutions, to determine the number of persons with Down syndrome who would have had earnings if they were not handicapped. This number was multiplied by the mean earnings of persons with income in that age group (Tab. 3, Part C). The total of these losses was then reduced by 5% to allow for that part of mean income that originates from transfer payments to disabled persons. In addition, among persons with Down syndrome who are living, the total was reduced by another 5% in order to allow for their limited earnings. This last adjustment assumes earnings of about $100 per month for persons with Down syndrome who are not in institutions. Given these assumptions, the estimated loss of output due to Down syndrome in 1983 was $2.6 billion.

Educational Expense

Almost all school-age children with Down syndrome receive special education services. The most recent and comprehensive information on the cost of special education was compiled by Kakalik et al. (10) for the 1977-1978 school year. This study shows that the costs of special education are substantial and vary among children with different handicaps and at different stages in their schooling.

Kakalik reported that it costs 3.34 times as much to provide special education services to a trainable, retarded child as it does to provide regular services. This represents the best estimate of the relative costs of educating a child with Down syndrome since the great majority of these children fall into the moderate range of mental retardation.

Average per pupil costs in the United States during 1983-1984 were estimated at $2,861. This estimate was developed by the U.S. Department of Education on the basis of data for the 1982-1983 school year. On this basis, the cost of educating a child with Down syndrome would, on the average, be $9,556, and the amount that should explicitly be attributed to Down syndrome would be $6,709 ($9,556-$2,861).

On the basis of data presented above, I estimate that there are almost 48,000 children with Down syndrome who are 5 to 19 yr old, of whom almost 4,000 are in large institutions. If we assume that all noninstitutionalized children with Down syndrome in this age range are attending school, the total cost of their education was $420 million during 1983, and the excess cost attributable to Down syndrome was $295 million.

The estimate could be refined by developing estimates broken down by age and level of mental retardation. However, this would involve many assumptions about the data, and the results, although methodologically satisfying, would merit no more confidence than those presented above.

Residential Care

We previously estimated that about 17,600 persons with Down syndrome are in large institutions or nursing homes. There are also approximately 60,000 mentally retarded persons living in small group quarters (1 to 15 residents) and foster homes. If we assume that the same percentage of these residents have Down syndrome as in large institutions, this adds about 6,000 persons with Down syndrome to the estimate of the number in residential care.

Bruininks (3) reported that an average per day per person reimbursement in 1982 was $86.23 for State institutions for mentally retarded persons, and $42.77 for group residences. On an annual basis, this amounts to $31,470 and $15,610, respectively. The lower cost for small group residences is partly due to the fact that they usually do not include the costs of day care and education services in their responses to the survey. These services are often provided by the institution and included in the institution budget. Ashbaugh and Allard (1) found narrower differences between the costs of institutional care and community-based care when account was taken of these additional costs. (We take account of the costs of education and day care elsewhere).

Since Bruininks' data were collected during 1982, we estimate the costs of residential care in 1983 by adjusting the 1982 costs by the change in the cost of living between 1982 and 1983. For 1983, estimated residential care costs are $32,810 and $16,270 for institutional care and group home care, respectively.

These measures of the costs of residential care include the costs of shelter, food, and other normal costs. A rough adjustment for these "hotel" costs is obtained by subtracting $3,600 from the annual cost of care in residential facilities. This is roughly the Federal payment for SSI beneficiaries who receive a full year's benefits.

On the basis of these data, costs of residential care in 1983 for persons with Down syndrome were $578,600 for institutions and $98,000,000 for group residences. Subtract from these costs our estimate of normal consumption expenses, and the excess cost of these forms of residential care comes to $514,000,000 for institutions and $76,000,000 for group quarters.

Day Programs

Many of the approximately 57,000 adults with Down syndrome who live in the community participate in day care and workshop programs. Bellamy and Buckley (2) estimated that during 1983-1984 there were 136,000 mentally retarded clients in day adult programs or work activity centers that received funding from State agencies for mentally retarded and developmentally disabled persons. There are, of course, other day care and work activity center programs that do not receive funding from these agencies. It is probable that there were approximately 200,000 or more mentally retarded persons in adult day programs, work activity centers, or sheltered workshops during 1983, with the great majority being in work activity centers or day programs. Many persons with Down syndrome are undoubtedly enrolled in these programs and we will assume that about 50% of the persons with Down syndrome in the community are enrolled in these programs (approximately 28,000 persons).

Bellamy and Buckley (2) also reported that the average expenditure by State mental retardation and developmental disability agencies was about $5,200 per year per person for persons in day programs or work activity centers. This is a conservative estimate of the cost of these programs since additional funds are supplied by local governments, private groups, and other State agencies, particularly vocational rehabilitation agencies. Assuming an average cost of $5,200 per year for adults with Down syndrome in day programs and work activity centers, the annual cost during 1983 for this type of care was $146,000.

Prevention

The most important method of preventing Down syndrome is to evaluate the chromosomes in the cells of a fetus during the second trimester of pregnancy and to terminate the pregnancy if Down syndrome or some other serious defect is identified. No national data on the number of amniocentesis procedures performed in the United States are available. During 1983, there were 10,919 amniocentesis procedures performed in New York State according to Dr. Ernest Hook of the New York State Department of Health. Dr. Hook estimates that the rate of amniocentesis procedures in New York State is 1.5 to 2.5 times the rate of the rest of the country (Hook, pers. comm.). On this basis, there would have been between 50,000 and 75,000 such procedures performed during 1983. This compares with a conservative estimate that there were over 35,000 amniocentesis procedures that was obtained from the Federal Center for Disease Control. On balance, it is reasonable to assume that 50,000 of these procedures were performed during 1983. The cost of the amniocentesis procedure is estimated to vary from $500 to $1,000 (Hook, pers. comm.; Smith, pers. comm.), including the ultrasound examination, drawing of the sample, chromosome analysis, and counseling for the patient.

If we assume that the average fee for amniocentesis was $650 and that 50,000 procedures were performed during 1983, then the estimated cost of these procedures would be $32,500,000. To this total should be added the additional cost of terminating the pregnancy, if indicated. However, only 66 abortions were performed in New York State after an amniocentesis procedure which would indicate 300 to 450 abortions nationally for the purpose of preventing Down syndrome. At a cost of $1,000 per abortion, this adds less than $500,000 to the cost.

Social Cost

The various components of the social cost of aneuploidy that we have been describing are summarized in Tab. 4. The total, a little over $3.6 billion, must be interpreted in light of our previous caveats about economic analyses. The data obviously contain many assumptions. In addition,

Tab. 4. Estimated social cost of Down syndrome, 1983.

Loss of output	
Among living persons	$ 841,000,000
Due to premature death	$1,749,000,000
Excess educational expense	$ 295,000,000
Excess residential care expense	$ 590,000,000
Adult day care	$ 146,000,000
Prevention	$ 33,000,000
Total:	$3,654,000,000

important components of costs were not measured. In particular, the value of homemaking services and other unpaid work was not included. A very conservative estimate of the value of these losses would increase our estimate of social costs by $900 million (about 1/3 the loss of market output) to a total of over $4.5 billion. Other types of costs that are not included are psychic costs, the costs of vocational rehabilitation services, and the costs of genetic counseling.

On the other hand, it is clear that Down syndrome is not a minor problem. Based on reasonable judgements about the data, we identified losses of about $3.6 billion due to Down syndrome that could otherwise be used to increase social well-being. If we included losses of the value of homemaking and other unpaid services, the social cost of Down syndrome would increase to $4.6 billion. Although this amount is dwarfed by magnitudes such as $200 billion Federal deficits, it still represents a substantial cost.

LIFETIME COST OF ANEUPLOIDY

Another way to look at Down syndrome is to examine the social costs involved over the lifetime of an infant born with the condition. We begin with a high-cost case of an infant with Down syndrome who will remain in his or her natural home until age 21 yr, and then be placed in institutional care for the rest of his or her life. We assume (obviously with some degree of error) that no unusual costs are involved during the first 4 years of life and that the individual will attend school from age 5 to age 21 yr.

Educational Costs

The following procedure is used to estimate the present value of these future school expenses, e.g., the amount that would need to be set aside at birth in order to cover these future costs. All subsequent estimates are based on 1983 prices. In labor-intensive industries, such as education and residential care, we normally expect productivity to show little change over time, i.e., we expect pupil-teacher and staff-client ratios to remain relatively constant. Persons who work in these industries will share in generally rising productivity through increases in their earnings. In consequence, the price of educational and residential care services should rise over time, in real terms, at a rate approximating productivity growth in the economy (since productivity will not change in these industries).

Despite economic fluctuations, individual productivity growth has been substantial in the United States. Between 1960 and 1982, the per capita Gross National Product (adjusted for inflation) grew by 2.4%; between 1965 and 1982, it grew by 2.1%; between 1970 and 1982, it grew by 2.0%. We assume that future per capita productivity will rise by 2% per year and that educational expenses per child, in real terms, will also rise at this rate. Based on the estimated annual per student cost of education in 1983, the present value of the future cost (discounted at 7%) of sending a person with Down syndrome to school from the ages of 5 to 21 yr are $90,000 and the excess cost attributable to Down syndrome is $63,000.

Of course, a substantial percentage of children with Down syndrome will die before completing school. The average cost of education per child born with Down syndrome (i.e., adjusted for mortality) was calculated by multiplying the present value of future costs of education at different ages downward by the percentage not expected to survive to these ages using the Hagard-Carter data (6) (Tab. 1). On this basis, the expected total cost of education would be $56,000 and the excess cost due to Down syndrome would be $39,000.

Residential Care Costs

Similarly, we assume that the costs of residential care will rise, in real terms, by 2% a year. Based on our previous estimate of residential care costs during 1983, the present value of the total cost of providing institutional care from age 22 to age 70 yr for a person with Down syndrome is $236,000. This is the amount, in 1983 prices, that would need to be set aside at birth in order to pay for residential care from the ages of 22 to 70 yr if there were no inflation. If we adjust this figure for the cost of normal shelter and housing costs (by subtracting $3,600 from the 1983 cost of institutional care), the excess cost of institutional care is $211,000. If we adjust for mortality, however, as we did in the case of educational costs, the estimated present value of future residential care costs falls dramatically to $83,200, and if we adjust for normal shelter and housing costs, to $74,600.

Suppose that persons with Down syndrome were placed in community residentical facilities and participated in an adult day program. In 1983, the average cost of these two services was estimated at $20,810. Assuming that this cost increases by 2% per year, the present value of this type of residential care from age 22 to age 70 yr is $140,500, and if adjusted for normal living expenses, $116,200. If these costs are adjusted for mortality, they fall to $49,600 and $41,000, respectively.

Earnings Loss

To estimate earnings loss, the standard assumption was employed that people born in 1983 would follow the same age-earnings profile over time as people living in 1983 as they reached the specified age levels, except that earnings would rise to reflect productivity change. Average earnings for all persons in different age groups (including persons without income as well as those with income) were calculated by multiplying the percentage with income times the average income of persons with earnings (Tab. 5). The present value of future income for persons born in 1983 was calculated by multiplying the average income of persons in each age group in 1983 (Tab. 5, Column 1) times the sum of the present value of a dollar (increased by the estimated amount of productivity change) in each of the yr

Tab. 5. Estimated lifetime earnings loss among persons with Down syndrome born in 1983.

Age (yr)	Average income ($)[a]	Adjustment factor for productivity change, discounting,[b] and years in age range	Percentage of general population surviving to specified ages	Percentage of persons with Down syndrome surviving to specified ages
15-19	1,591.85	2.22148	97.862	57.5
20-24	7,045.33	1.74873	97.285	52.0
25-34	13,267.61	2.46024	96.293	40.0
35-44	17,098.85	1.52453	94.523	33.0
45-54	17,153.54	0.94470	90.390	23.0
55-64	15,002.30	0.58542	81.492	11.0

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[a] Includes both men and women, and averaged over persons with and and without income.

[b] The adjustment factor was derived as follows. Assume a dollar increases by 2% per yr (productivity change), and is discounted at 7%. Fifteen yr later, the present value of this dollar would be about $0.48, and will decrease slightly each succeeding yr. If these present values are summed for the 15th through the 19th yr, the total is about $2.22, and if summed for the 20th through the 24th yr, it would be about $1.75, and so on.

in the future age interval (Column 2; this adjusts these future earnings to present value and takes into account the number of yr in each interval) times the average probability of survival in that age range in the general population (Column 3). Normal survival rates were used since the loss of income among persons with Down syndrome was based on what persons who do not have Down syndrome would receive. The present value of this future income is $93,300.

As noted above, part of this income represents transfer payments for disability. Reducing the total by 5% to allow for these disability transfer payments leaves a net estimate of the present value of future income for persons born in 1983 of $88,700.

In order to estimate economic losses over the lifetime of a person with Down syndrome, we added together our estimates of income losses (other than disability transfer payments), the excess costs of institutional care, and the excess costs of educational services.

In the case of a person with Down syndrome who lives to age 70 yr and spends his or her adult life in institutional care, lifetime costs are $363,000 (earnings loss of $89,000, excess residential care cost of $211,000, and excess educational cost of $63,000). If we adjust for expected mortality, lifetime costs decline to $203,000 (earnings loss of $89,000, excess residential care cost of $75,000, and excess educational cost of $39,000). These amounts may appear low relative to similar estimates that have been made in the past. This is entirely due to discounting future costs to present values. In absolute, undiscounted terms, the value of productivity losses and treatment costs in this worst-case scenario would amount to over $2 million (1983 prices) over the person's lifetime.

This worst-case scenario, although typical of such estimates, overstates the costs that are likely to be incurred. One reason is that increasing numbers of severely mentally retarded persons are being placed in small group homes in the community. Institutional care may some day be virtually eliminated. If we assume that persons born with Down syndrome spend their adult lives in a community-based residential facility and are placed in adult day care, the lifetime costs of aneuploidy are reduced to $268,000 or to $169,000, depending upon whether or not an adjustment is made for mortality.

A second reason why the worst-case scenario is too high is that few severely mentally retarded persons spend their entire adult life in residential care. Based on our earlier estimates, it appears that about 1/4 of adults with Down syndrome are in residential care. Apparently, most persons with this condition live with relatives. However, rather than assume that persons with Down syndrome will spend, on the average, only 1/4 of their lives in residential care, we believe that it is more reasonable to assume that, on the average, they will spend about 1/2 of their lives in this form of care. We expect that increasing numbers of mentally retarded persons will live in residential facilities as the availability of community-based facilities increases. This assumption further reduces our estimates of lifetime costs to $210,000 and $149,000 depending upon whether an adjustment is made for high mortality and assuming that community-based residential care is utilized.

A third reason why the worst-case scenario overstates the economic costs of Down syndrome is that many people believe that moderate and even severely mentally retarded people, including persons with Down syndrome, can engage in substantial productive work if given appropriate services and if placed on a suitable job.

We will assume that persons with Down syndrome will be able to earn about 10% of the income that they would have been expected to receive during their lives if it were not for their handicap. Some people will regard this as conservative, and some as optimistic. This reduces our estimates of the lifetime costs of Down syndrome to $200,000 in the case of a person who lives to age 70 yr, and $145,000 if we adjust the data for mortality among persons with Down syndrome (this assumes that persons with Down syndrome will spend about 1/2 of their lives in community-based residential facilities).*

These last figures represent conservative, but reasonable, estimates of the lifetime costs of aneuploidy. Obviously, these estimates will be affected by many variables, e.g., a decrease in mortality, greater productivity than anticipated, and changes in the standards of care. Moreover, they measure only part of the costs that will actually occur. For example, no adjustment has been made for unusual medical expenses associated with Down syndrome. Not only do persons with Down syndrome have a high rate of physical problems, but they also appear to have problems associated with aging earlier than the general population (12). It should also be noted that these costs will vary substantially among persons with Down syndrome since there is great variation in the costs of educational and residential care services among different jurisdictions and facilities, because persons with aneuploidy will differ in the extent that they use these services, and because productivity will vary among persons with Down syndrome.

COST-BENEFIT ESTIMATES

The benefits of preventing a case of Down syndrome will equal the lifetime costs that were estimated in the previous section if a normal child is born to replace the one that would have had Down syndrome. Although we will limit our analysis to the replacement case (which assumes that a family predetermines the number of children that it wishes to have), a rough approximation to a benefit-cost ratio for the nonreplacement case (where families have one less child for each case of Down syndrome averted) could be made by reducing the benefit-cost ratio for the replacement case by about 1/3.

In principle, the benefits of preventing Down syndrome should be measured by lifetime costs that are adjusted for excess mortality among persons with this condition. We will, however, continue to make 2 sets of calculations: one that assumes that persons with Down syndrome will live to age 70 yr, and one that adjusts for the unusually high mortality that exists among persons with Down syndrome. We do this partly because we recognize that our information on mortality among persons with Down syndrome must be regarded as tentative. In addition, mortality rates among these persons are declining, which will have the effect of increasing lifetime costs and the benefits of prevention. In consequence, the true benefit-cost ratio will be somewhat higher than indicated by our calculations. Finally, some people, particularly prospective parents, probably have a strong interest in calculations based on the assumption of a long life for persons with Down syndrome. In any event, it is distasteful to attribute a benefit to premature death, as is inherent in these calculations.

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* This adjustment resulted in approximately a $4 million reduction in lifetime costs if lifetime costs are adjusted for mortality, and about a $10 million reduction if it is assumed that a person with Down syndrome lives to be age 70 yr. Since mortality rates are decreasing, this probably represents a slight overstatement of lifetime costs when they are adjusted for mortality on the basis of the Hagard-Carter data.

What does it cost to prevent Down syndrome? In some cases, costs can be virtually nonexistent, e.g., counseling to explain the advantages of having children before age 35 yr. We should note that Edel and Edel argue that there is a substantial economic cost to women who choose to have their children while young and thereby defer the progress of their careers (Edel and Edel, this Volume).

Most prevention efforts, however, take the form of identifying fetuses with Down syndrome during the second trimester of pregnancy and aborting if indicated. The major cost in this form of prevention is the cost of identifying the fetus with Down syndrome. We previously estimated that an abortion will cost approximately $1,000 and that an amniocentesis procedure (including counseling and withdrawal of fluid) will cost approximately $650. The additional piece of information that is needed is the number of pregnant women that must be screened in order to identify a fetus with Down syndrome.

One way to compare costs and benefits is to determine the breakeven point in terms of the percentage of cases that must be found as a result of amniocentesis procedures in order for benefits to equal costs. This can be calculated by dividing $199,000 and $144,000 (roughly the savings from preventing a case of Down syndrome aneuploidy less the price of an abortion [the lower figure is adjusted for mortality]) by $650, the price of an amniocentesis procedure. This calculation indicates that the breakeven point is reached or exceeded as long as at least one case of Down syndrome is detected out of every 306 or 222 examinations. This amounts to a case-finding rate of either 0.33%, or 0.45%. The higher breakeven point, 0.45%, which is based on mentality-adjusted data, is, of course, the theoretically correct rate.

Data from Massachusetts, New York, and Sweden (8) indicate that at a maternal age of 36 yr (at birth) the prevalence of aneuploidy is about 0.33% rising to about 0.43% at age 37 yr and to about 0.56% at age 38 yr. These data indicate that the benefits would equal costs if all pregnant women above the age of 36 or 37 yr were screened for Down syndrome if they would agree to abort fetuses found to have this condition. The incidence of Down syndrome rises rapidly with maternal age: its about 0.9% at age 40 yr, indicating a ratio of benefits to costs of between 2 and 3, and rises dramatically to about 8% at age 49 yr, indicating cost-benefit ratios of 17 and 24, depending upon which measure of benefits is preferred.

There are other groups at high risk of having a child with Down syndrome, e.g., persons who have previously given birth to a child with aneuploidy and parents who themselves have aneuploidy, for whom the benefits to costs ratio of preventing Down syndrome will obviously be very high.

Do these estimates indicate that amniocentesis should not be done if the case-finding rate is less than 0.33 or 0.46%? By no means. Clearly, we have not identified all of the benefits of the amniocentesis procedure. Types of birth defects other than Down syndrome would be identified and prevented. In addition, only part of the benefits of preventing Down syndrome could be measured. If account were taken of these factors, the benefit-cost ratio would be higher and the corresponding breakeven point in terms of the case-finding rate would be lower.

Conversely, does a positive benefit-cost ratio for screening a particular group of people mean that amniocentesis is always justified? Again the answer is no. As we have emphasized, there may be psychic costs to aborting a fetus which, in some instances, outweigh any benefits that might be derived.*

Although economic analyses may provide interesting and important insights into the costs and benefits of preventing Down syndrome, decisions as to whether prevention will actually take place must ultimately be left to prospective parents who will probably use very different criteria in making their decision, and by society, which is still debating the issue of whether abortion is morally permissible; if it is not, the costs and benefits that we have estimated are not relevant.

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* We should observe that a few cases of Down syndrome will be identified and aborted during the second trimester of pregnancy that would have spontaneously aborted had the pregnancy not been terminated. However, the additional cost involved will amount to only a few hundred dollars per case of Down syndrome prevented and is within the rounding error in our estimates.

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Editors' Comment: The above discussion pertains to Down syndrome, certainly the greatest but not the only burden imposed by chromosome abnormality. As discussion elsewhere in this Volume indicates, there are numerous other consequences of aneuploidy, although their economic impact is much more difficult to estimate. Whatever the exact costs may be, these estimates indicate that research devoted to primary prevention of both numerical and structural abnormalities is well merited on economic grounds if not social ones.

ECONOMIC COSTS OF ANEUPLOIDY: SOME PROBLEMS OF MEASUREMENT AND CONCEPTUALIZATION*

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INTRODUCTION

This chapter analyzes some problems in measuring economic burdens of aneuploidy. Examining the costs of aneuploidy requires an appropriate means of measurement or estimation. We reviewed studies in the light of debates over cost-benefit analysis, and suggest that some burdens have been misspecified or omitted (e.g., Ref. 32 and 36). We then suggest that if they are properly accounted for, reduction of aneuploidy risks can be shown to have major economic benefits. This suggests the importance of research directed toward reduction of such risks.

We review first the attempts made so far to examine the costs of aneuploidy, along with a few relevant attempts to evaluate the costs of other medical conditions. This will then allow us to examine what factors excluded from these analyses may be added to yield more useful results.

If children were commodities, produced for public or private sale like bus rides or dolls, it would be a simple matter to evaluate the cost of a birth defect. One would merely need to examine the market prices of "normal" and "abnormal" babies as they were delivered from whatever enterprise provided them, and note the difference.

It might be tempting, in the light of the incipient (if sublegal) development of a "market" in surrogate mothering, to judge the price of a "normal" infant by the price parents are willing to pay for them. But comparing the $20,000 or so that some parents seem willing to pay with a zero market value for "defective" merchandise would provide us with a fallacious estimate. The $20,000 figure is perhaps valid as an estimate of the value of the labor (in both senses) of the surrogate, adjusted upward for medical and legal risks, stigma, agents' fees, and the like. Were it not for the legal problems, the price would be lower. After all, there are also many free adoptions. But this does not mean that $20,000 is the upper bound estimate of the value of normalcy, for an infant with a birth defect is not merely a piece of unsold merchandise. As a living individual it is subject to suffering itself; as a member of society or family, it has entitlements

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* This research was partially supported by grants from the P.S.C./B.H.E. Faculty Research Award Program of the City University of New York.

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to resources for its care. Thus, from the buyers' viewpoint, it does not merely lack positive value. In violation of the normal laws of economics, it has a negative value. (From the social viewpoint, one would say the cost of the defect is equal to the $20,000 or fraction thereof that is the market price of a normal infant, plus the cost of the negative value to the responsible caretaker, plus whatever value one might put on the suffering of the infant itself, and of other empathetic parties).

All of this sounds forced and cruel, as economic analyses of human conditions so often do. But it situates the analyses that have been done of costs of Down syndrome accurately enough, in that these have generally taken only the measurable costs of caretaking as the cost of the defect. In simplest studies (e.g., Ref. 34), the cost is taken to be the discounted cost of lifetime institutionalization. In more complex studies, the probabilities of different degrees of retardation are factored in, with expected costs of institutionalization and alternative forms of home care used depending on the degree of retardation. In addition, the comparison of the Down syndrome child and the normal child is made through consideration of the difference in the discounted value of what each is expected to earn over its lifetime. Adding the care and lost earnings costs gives the value of the disorder. This approach holds both in Conley's study (8) of the overall economic impact of retardation, and in the numerous other studies of Down syndrome and genetic screening (1,7,9,17,18,20,23).

We shall not dwell on the details of these studies. They differ somewhat in their method of estimating institutionalization and home care costs for a Down child. However, they are relatively consistent in their estimates of the discounted value of an individual's care, citing such costs as falling in the range of $100,000 to $200,000 in early 1970s prices. The variation depends in part on the average lifespan estimated, but this is not a major consideration since, due to use of a discount rate, effects more than a decade or two into the future cannot affect the result much. The discount rate is itself a more important source of variation, as is the means of estimating annual institutionalization costs. The assumption on the proportion institutionalized is also variable, with Stein et al. (35) assuming all cases institutionalized, but with most others assuming a mix of institutionalization and home care. Home care costs are estimated roughly, and different means of treating IQ and other evidence of Down syndrome's effects lead to different assumptions about the limited earning power of young adults with Down syndrome. In two cases explicit account is made of the probability that parents will incur losses of income while staying home to care for Down children. Andreano and McCollum (1) assume that the mother's labor force participation and earnings are reduced by half; Hagard and Carter (18) assume a smaller reduction.

We may pause briefly here to consider the appropriateness of costs of care and lost earnings as a decision variable for public policy purposes. The more salient ethical precept is one concerning the social valuation of handicapped individuals. The presumption is that the institutionalized Down child has only negative value; that even a noninstitutionalized and partially employable individual may be treated as having negative value as long as that individual's work will never cover his or her upkeep costs. This is the same assumption that was presented in studies of birth-control for third-world countries, which suggested every birth not prevented represented a net cost to the countries involved (13). One difference is that in the case of Down syndrome, the assumption might be made that the individual not born might be replaced by another normal birth, rather than prevented outright.

This rather stern valuation procedure might be justifiable from one of two possible perspectives. One is from the viewpont of a national well-being, defined so that costs of institutionalization and even of lessened earnings of child and mother are seen as draining resources from a national effort to foster economic growth or maximize otherwise usable GNP. This sort of perspective might be more tolerable in the context of a crash industrialization program of the sort presumed in the studies of third-world birth control than it is for the United States today. The other is the perspective that the analysis does somehow reflect a consensus shared by society and by the potential parents of Down children that the costs do represent real burdens. This aspect will be considered later.

The costs (of institutionalization and care, and of foregone earnings) estimated as we have shown are compared in the existing studies of Down to costs of avoiding those burdens through amniocentesis and the option of abortion. Estimates from institutional sources are taken of the specific costs of amniocentesis and abortion. These costs, individually, are small and may be taken as reasonably reliable (see Ref. 1 for some qualifications). What is highly variable, however, is the probability that any given amniocentesis will discover a case of Down syndrome. If it could be known ahead of time which cases would have aneuploid conditions, the costs of a confirmatory amniocentesis and abortion could be compared directly to the costs of avoiding a "defective" birth. But if so, there would be little real need for amniocentesis. The procedure is used because there are probabilistic risks.

Thus for each positive amniocentesis done there will be many negative ones. The relationship between amniocentesis and births prevented will be mediated by probabilities of the existence of aneuploidy at the time of the amniocentesis, by rates of natural fetal loss of aneuploids between then and birth, and by probabilities that parents will in fact opt for abortion if results are positive. The initial studies did not correct for the latter 2 factors, but they have been factored into the more recent studies. The initial probability of chromosomal abnormality is the remaining factor, and it is worthy of discussion.

Amniocentesis is clearly cost-effective in the case of hereditary disorders like Tay-Sachs disease, for which certain parents have known high risks. (This is at least true if parents wish to know if fetuses are affected). But most types of aneuploidy are not inherited from parents who are obvious carriers, and in the population at large, incidence rates are relatively low. Thus mass screening of a random population would not discover enough cases to "pay back" program costs, according to the existing cost-benefit studies. Only for higher-risk subgroups than that of all pregnant women is the cost justifiable from an aggregate benefits perspective. Thus attention has been turned to the one very visible factor which has an established, simple (i.e., nonmultivariate) correlation with risks: that is, maternal age.

The cost-benefit studies we have surveyed have used several different studies as their basis, from among the large literature of surveys which estimated risks of Down syndrome by 1- or 5-year maternal age groups. Since these studies will report higher risks at higher maternal ages, it is not surprising that amniocentesis is seen as a more effective screening process, in terms of cases caught per dollar spent, at higher ages. Indeed, despite all of the variation in other assumptions made in the different studies, the results appear to be driven by the similarity of the age-risk data used. In all cases, the benefits of avoiding a Down birth outweigh the detection and abortion costs for women in the over-40 yr age group, but are outweighed by the costs for the lower-risk, under-30 yr age group. The studies vary slightly as to the cutoff age at which benefits pass costs. Two different criteria of passing may be used: an average benefit criterion in which it is asked whether benefits will outweigh costs if all women of age X and above are tested, and a marginal benefit criterion, in which it is asked whether total benefits will rise or fall if the cutoff point is reduced by a year. In general the average benefit cutoff turns out to be somewhere in the low to middle 30s and the marginal benefit in the middle or perhaps the upper 30s.

This finding appears to mean that for some women, provision of amniocentesis can be justified to health plans and public agencies as socially cost-saving. What is more, the studies appear to provide some scientific validation for the choice of particular cutoff ages above which amniocentesis will be covered (in the absence of any other genetic evidence), and below which it must be left to the private market decision of the mother. We will argue, below, that the very statement of a cutoff age may have social costs. For the moment, however, let us consider the sensitivity of the cost-benefit analysis itself to the data on age and risk.

We recognize that a strong consensus exists among epidemiologists that there is a correlation (and hence, presumably, a relationship) between maternal age and trisomy 21, which is usually associated with Down syndrome, and that an age-related incidence rate is found in the less voluminous studies of some other trisomies. Further, some studies have attempted to sort out other factors such as father's age, race, location in the world, and certain limited socioeconomic variables (see, e.g., Ref. 22). Except where separate national studies have been compared (e.g., Ref. 24) these studies have involved small samples, especially compared to the major studies of maternal age. A large sample study which considered all aspects of mothers' past histories, including environmental exposures and prior reproductive history, and socioeconomic conditions, has not been undertaken.

This is unfortunate, because at least until recently, advanced maternal age has not been independent of socioeconomic status. Some possibility exists that maternal age may itself have been correlated to experiences (like diet or environmental exposures) that might affect the general process of aging. As a result we must consider as open the possibility that mother's age in part serves as proxy for other factors. Furthermore, the lack of consensus on whether the age relationship simply measures effects of cellular aging or whether it results from the cumulation over time of environmental exposures is troubling. This is particularly so since there is a degree to which results of individual surveys are corrected to make them conform to other studies (e.g., the use of a markup figure for underreporting in the March of Dimes New York Study), so that comparison between studies in presumably different environments, like New York and Sweden, is left problematical. We do not, in short, really know what the maternal-age effect measures (c.f., Ref. 23 and 24).

Now, for some purposes this may not matter. If the purpose of measurement is to argue that benefits will exceed costs if all women over 40, 35, or maybe 32 yr are screened for aneuploidy, then it doesn't matter why the curve has its present shape. We only need to observe that, like many cost-benefit studies, the analysis of amniocentesis takes the state of the world as a given. Results might, or might not, be somewhat different if environmental factors got a lot better or a lot worse, or if older women suddenly started marrying younger men in large numbers. But if the studies are to be used in ways that go beyond their original purpose, to consider the costs and benefits of environmental changes or to consider the impact of age criteria, then truly multivariate studies would be needed. Indeed, a call for such studies would be one of our main policy conclusions.

STIGMA AND SOCIAL COSTS

As the previous section indicates, prior studies of the burdens of

Down syndrome have taken custodial costs and lost earnings to represent its social costs. Costs of other syndromes associated with aneuploidy have not been estimated at all. This results, we would argue, in a severe underrepresentation of the costs of aneuploidy. This section and the next discuss some previously omitted costs. Although previous studies have mentioned personal feelings of victims and their families as costs, they have tended to downplay them as uncertain and unquantifiable. This section will argue that the stigma placed on Down syndrome and other syndromes associated with aneuploidy cause major and potentially measurable costs. Similarly, although Andreano and McCollum (1) cite as a cost anxiety by expectant parents, and consider damage to careers of mothers of Down children, they do not cite an even more important indirect cost: distortion of careers due to anxiety about having a "detective" child. The following section will consider this effect, and attempt to develop a measure of the indirect costs of Down syndrome using labor market observations.

Consideration of the social costs created by stigma requires attention to present attitudes toward Down syndrome, and to other syndromes that may result from aneuploidy (e.g., Klinefelter). The effects of stigma attached to diseases are frequently mentioned among potential costs for consideration in cost-benefit analyses. Indeed Andreano and McCollum (1) give considerable attention to those costs in the nonquantitative section of their paper on Down syndrome, only to exclude them from the quantitative portion as too difficult to estimate. Such an exclusion of costs, because they are not easily measurable, is frequent in cost-benefit analysis, and may make such studies grossly underestimate true social costs (36). In one study, even a very modest estimate of stigma costs accounted for over 40% of the potential benefits of disease eradication. This was Klarman's estimate (25) that the embarrassment of a history of syphilis, or the need to avoid jobs with disclosure requirements, might reduce future income by 1%. Even this minimal estimate implied an annual loss of $1.3 billion dollars, almost equivalent to losses from medical and time costs of treatment.

In our society, retardation, deformity, sex, and childbearing are all emotionally charged topics. The stigma to both an aneuploid individual and that individual's parents (in the case of a visible birth defect associated with retardation) may be great. Our research, for example, has suggested that women are significantly more disturbed by the prospect of a Down child than a child with a heart defect. A majority of our New York City sample felt that having a "mongoloid" child was the "worst thing" that could happen in a pregnancy. And in a question asking under what conditions the respondent would consider abortion, over 60% said they would seriously consider abortion if they knew they were having a Down child. Almost none would consider abortion for heart defect.

The response to this question (backed by both in-depth interviews and additional survey questions) indicates a high degree of stigma attached to birth defects and, in particular, to Down or retarded children (c.f., Ref. 37). For many women in this society the production of a child is seen as a proof of "womanliness," evidence of self-worth through the ability to produce a "perfect" child. There is a tendency to "self blame" if one's offspring in later years does poorly in school, uses drugs, etc. The mother of a child with a disability is apt to see herself as "at fault," rather than to analyze the contributing proportion of social structure or chance. The guilt and stigma generated by seeing oneself, and being seen by others, as the mother of a "defective" child, is an important source of stress.

The increased stress from a Down child is potentially very costly. Stress is known to have numerous physical side effects, high blood pressure among them, which would show up in increased medical costs. Nor is the "purely emotional" side of the burden unmeasurable in principle. Virtually all families with "disabled" children would benefit from professional counseling, and many families probably need ongoing therapy. A typical cost for an hour of therapy in New York City is $80. Both the pyschological and physical costs should be calculated when doing cost-benefit analysis, but studies have not done so. Even making a very minimal assumption of 25 hours of therapy would add $2,000 to per case costs.

Nor can one limit the family members who may need long term medical or psychological aid. Both parents and all siblings come under the stress and may need help. Further, the internal and external stresses tend to prove difficult for many marriages and the divorce rate is higher in families with Down children (16). This puts both additional stress and extreme economic pressure on the family, probably necessitating additional outside aid, if there is an extended family. While some internal stress may be alleviated, it may also mean that there is an extension of costs. In other words, a grandmother may be able to alleviate some stress by listening, or lessen some costs by babysitting, but she in turn may be subject to stigma from having a "Down" grandchild. And the costs of outside help necessary to her, whether or not she can afford it and hence obtain it, need to be included in any analysis.

Apart from the direct costs of coping with stress, there may be other indirect costs brought on by stigma that parallel the employment costs that Klarman (25) posited in the case of syphilis. Some of these can only be guessed at, given available information, but may be considerable. To the best of our knowledge there have been no studies asking if rent increases for families with disabled children. It would hardly be surprising in a rental market in which 25% of all housing stock refuses all children (26) to find that families with visibly disabled children had greater difficulty and had to pay not only higher costs in terms of physically altered shelter (e.g., ramps in lieu of stairs), but discovered that they were denied equal access to even ordinary housing, necessitating higher rents.

Another extremely important cost may be the loss to a family of neighborhood ties, if neighbors either reject the entire family or the child. There may be an emotional loss. There are greater costs from not having "safe" houses for a child in trouble to go to if the parents are not present. One can calculate the number of hours neighborhood children spend at other houses (and hence the time for the parent at home to clean, breathe, recuperate emotionally, or participate in paid employment). Disabled children are frequently excluded and this is particularly true of Down children who are frequently perceived as too "different" and hence stigmatized.

One aspect of family costs has been included, at least to a token degree, in previous studies. Most disabled children take additional "care," both in terms of medical and educational attention, and in terms of hours of care by family members. One of the many sources of stress that may befall families is that just as more medical expenses must be incurred, there will be a loss of income as one family member or another reduces working hours or leaves the labor market in order to provide care. Andreano and McCollum (1) have made some correction for this by assuming that in addition to medical costs for noninstitutionalized Down children, there will be a cost equal to a 50% reduction in earnings by the child's mother. It is noteworthy that no earnings reduction is imputed to the father, not even a 1% loss which was assumed in Klarman's study of venereal disease (25). The Andreano-McCollum adjustment is certainly better than the complete absence of attention to this "unmeasurable" aspect of family-norm costs. But we would argue that the amount estimated is too low to cover all of the psychological and medical costs to parents resulting from stress and stigma, all of the possible housing and neighborhood costs stemming from discrimination, or even all of the job-related costs for parents.

While we have no direct measures to offer, we will suggest in the next section that the costs families (or, within families, women) bear to reduce risks of Down children suggest a very high valuation on these costs.

Also, the costs of stigma, stress, and discrimination of other syndromes associated with aneuploidy have not been measured. Although not as visible in their manifestations as Down syndrome, some of the aneuploid conditions, such as Klinefelter syndrome, involve problems of sexual maturation. Given the tremendous importance of sexual identity in our society, the stresses to the individual, and the consequent needs for counseling and other treatment may be considerable in these cases. Other aneuploid conditions, such as Patau syndrome, result in an extremely short lifespan. Although from the perspective of most of the cost-benefit studies, that these cases at least have the virtue that suffering and institutionalization costs are brief, parents will still suffer the stress and stigma of having had a "defective" child. What is more, given present pressures on the medical community to prolong life in marginal cases, both hospitalization costs and the stress (and sometimes the legal costs) of decisions over medical care may be elevated. At any rate, it is clear that the costs of Down syndrome presented in the previous studies do not exhaust the economic burdens of aneuploidy.

ANEUPLOIDY RISK AND WOMEN'S EARNINGS

There is one more area of costs of aneuploidy that needs examination. The most important of the added costs, in our present economic system, may be a loss in earning capacity, not of the aneuploid individual, nor even of the parents of the affected individual. Rather, women in general may have their labor market opportunities and behavior distorted by the attempt to avoid birth defects in potential offspring. Two particular examples of this are apparent. The first concerns possible job discrimination on the basis of environmental risk; the second involves labor market consequences of childbearing age.

There has been considerable discussion of proposals by employers to screen individuals working with hazardous materials for genetic susceptibility to those materials, or to prevent women of childbearing age from working with materials which might prove hazardous to their offspring. The cases that have attracted the most attention have not involved aneuploidy itself, but rather Mendelian conditions like sickle cell disease. Since we do not know with assurance if any chemicals do induce aneuploidy, this is not surprising. But the experience of attempts to restrict work by those with sickle cell disease or other hereditary conditions, or to restrict work by pregnant women for reasons other than fear of aneuploidy, is suggestive. It demonstrates what could happen if specific chemicals or radiation were to be implicated as risk factors for aneuploidy. Conditions like sickle cell for which incidence is correlated to race, and those like pregnancy which are sex-specific, can be used as markers which create employment barriers for labor-market disadvantaged groups. While the attempt to reduce damage to individuals is laudable, a public policy analysis must consider also the impact of removing employment opportunities. This issue has been commented on at length, and the ethical issue of whether people should be barred from certain jobs for their own protection will not be resolved here (see Ref. 4 and 30). But it does suggest that if one were looking at the costs of some environmentally influenced condition, part of the economic cost would be the earnings foregone by those whose employment is limited because of the hazard.

A similar situation occurs because women try to avoid bearing Down syndrome children. Since there has been so much publicity about correlation between maternal age and Down risk, women frequently try to reduce risk by planning childbearing at earlier ages. Indeed, our research on childbearing age decisions suggests that risks are frequently reported to women, by the media and even by physicians, as being greater than the incidence rates found in the epidemiological literature. We have collected numerous cases of doctors informing people that "older" mothers (defined as over 40, over 35, or even quite frequently over 30 yr) "probably will" have "defective" children. As a result of this, and of other social pressures against "older" motherhood, women adjust their childbearing decisions. At present there is a strong social norm that a woman "should" have her first child by the time she is about 25 yr. Exaggerated reports of the risk of Down syndrome or "birth defects" generically are among the cautionary tales that enforce the norm (see Ref. 11, 12, and 33).

The problem is that in many occupations, childbearing in one's 20s instead of one's 30s greatly affects occupational success. Studies of certain high-level occupations like medicine, law, science, and corporate management suggest that the only women who reach top positions are those who remain childless or who have children after their careers are well-established--in their mid-to-late 30s at the earliest (3,5,14,19). Whether this is generalizable to a wider range of occupations is difficult to ascertain, but it would tentatively appear so. Mincer and Polachek's studies of statistical determinants of women's wages suggest a long gap in a work history does have negative consequences for future wages. At least among certain educational levels a gap earlier is worse than a gap later (27,31). Moore and Burt (29) find that teenage pregnancy has severe consequences for later wages, but do not distinguish the ages.

Our own attempt to examine wages in a large sample survey has involved use of the Panel Study of Income Dynamics, a sample of over 5,000 families. In the ninth wave of that study, indepth information was gathered from wives as well as heads of households in the sample, allowing a pooling of data on married and unmarried women. Taking the wages of mothers ages 40-59 yr in 1976, and grouping them by age at first childbearing and educational level yielded the results in Tab. 1. Among both college-educated and noncollege-educated women, those who had had their first children at ages over 30 yr were, by the time they were over 40 yr, receiving higher hourly wages than those who had had children in their 20s. Wages of those who had had children in their teens were even lower (12).

These wage effects are not unknown to women in career tracks. Indeed, we find that the choice between continuing a career and interrupting it for childbearing is felt as a harsh one by many women, some of whom go through periods of psychic distress as a result of nearing the social marker ages of 30 or 35 yr (6,10-12,15).

Tab. 1. Wages of mothers (in ¢/hr, 1976) by age at first childbearing and education. Sample size, n, in (). Data from 9th wave of Panel Study of Income Dynamics, analyzed in Edel and Edel (12).

Age at first childbearing	All educational levels		Some college		No college	
	¢	n	¢	n	¢	n
12-19	340	(160)	415	(6)	332	(154)
20-24	366	(175)	500	(34)	334	(141)
25-29	376	(135)	475	(33)	344	(102)
30-34	402	(60)	522	(16)	358	(44)
35+	466	(53)	575	(14)	427	(39)

At first it may appear that this tradeoff is unavoidable, given the epidemiological statistics. This is, however, too fatalistic a view. In the first place, changes in the structure of professions and other job ladders could be instituted which would make the employment side less unfavorable for women opting for younger motherhood. (We recommend the search for such reforms to those who wish to reduce incentives for abortion.) But even on the side of risk reduction, the situation is not hopeless. In the first place, prospective mothers' decisions are rarely made on the basis of perfect information about risks. Risks are perceived as greater than they are. Most particularly, it is frequently believed that Down syndrome can only occur if mothers are beyond some certain cutoff age, and that it is very likely beyond that age. Better information, even on current epidemiological work, would help. Women might then not regard the differences in risks at ages of, say, 28 and 33 yr, as being so significant as they now think they are.

This suggests that cost-benefit studies, which calculate a cutoff age for genetic screening, may themselves have high costs. Emphasizing a cutoff age may lead women to overestimate risks at higher ages, and to underestimate them at lower ages, and hence, perhaps, to distort their childbearing and career plans.

The possible magnitude of these labor market effects may be demonstrated by a few illustrative calculations using the figures from Tab. 1. We hypothesize two alternative career paths as the options that a woman is considering at age 24 yr. In the early-pregnancy path case, she drops out of the labor force at age 24 yr, and returns to a career 7 years later, immediately earning $3.50 per hour (in 1976 prices). In the late pregnancy path case, we assume a wage of $3.00 at age 24 yr, rising 10 cents a year, until she drops out at age 38 yr, to reenter again after a 7-yr gap. This time, she can earn $4.50 an hour. These hourly figures are slightly below the figures ($4.66 and $3.66) for these ages in Tab. 1, but the gap is the same. Different interest rates are assumed; a 2,000-hr workyear and a 40-yr time horizon (i.e., to age 64 yr) are presumed. The results are summarized in Tab. 2, and indicate a discounted value of the earnings differential of $34,458.98 with a 10% real discount rate, increasing by over $10,000 if the discount rate is 6% and decreasing by over $5,000 if the rate is 14%. This suggests, to put it most generally, that many women are paying the equivalent of $30,000 or more to reduce Down risks.

Tab. 2. Present value of future earnings for women 24 years old (1976) under different assumptions about childbearing age and discount rate. Early path implies childbearing at age 24 yr, late path at age 37 yr. In both cases a 7-year absence from work is presumed, otherwise fulltime work is estimated at 2,000 hr per year. Data on wages from Tab. 1 as explained in text. We thank N.K. Edel for programming help.

Discount rate (%)	Early path	Late path	Difference
6	70,902	115,841	44,939
8	51,112	89,936	38,824
10	37,969	72,427	34,458
12	28,927	20,107	31,180
14	22,514	51,114	28,600

This analysis suggests, also, an approach to the measurement of the costs of Down syndrome and other birth defects which could stand as an addition or alternative to the institutionalization costs and lost earnings approaches discussed earlier. If we take the original notion suggested for benefit and cost measures, namely willingness to pay, the figures in Tab. 2 present a measurement that may be more relevant than the hypothetical case of paid-for surrogacy used above. Table 2 implies that at least some women seem to be willing to pay $30,000 for a reduction in risk of a Down syndrome child. But this is not a payment for the difference between a certainty of Down syndrome and a certainty of normalcy. It is a payment for a further reduction of what is already (even at age 37 yr) a fairly low risk, about one in 400. The implied negative value of the certainty of Down syndrome might thus be considerably higher than $30,000.

Taking the 10% discount rate figure and making the extreme assumption that behavior obeys the postulates of rational expectations, we make the following calculations. According to Hook (21), the risk of Down syndrome at age 24 yr is in the range of 0.7 to 0.9 per 1,000 livebirths; at age 37 yr, the range is 4.1 to 6.4 per 1,000. Taking the midpoint of each range, we find the woman has been able to reduce risk from 5.25 to 0.8 per 1,000. In other words, risk is reduced by 4.45 cases per 1,000, or one in 224 births. Looking at it in another way, the expected value of removing one Down case is equal to the value of 224 career postponements (or 224 times $34,458.98). On these admittedly extreme assumptions, then, there are at least some mothers acting as if the negative value of a Down case were $7,028,792.

Obviously, not all mothers are in this situation. If we assume that the other extreme is mothers who do not place any negative value on Down syndrome children, and if we also assume a straight-line demand curve, the average value is halved, to just over $3,500,000. This estimate of the negative value is considerably above the values of about $100,000 or $200,000 estimated by all of the previous studies.

One should not take the figure of $3,500,000 per case as exact. Childbearing decisions, as we have noted, are affected by exaggerated notions of risk. The entire expected value approach may not be appropriate if decisions are based on a risk-minimization or minimax criterion. But our exercise does suggest that the figures that have been estimated thus far for the value of avoiding Down syndrome are too low, even if not necessarily by a factor of 20 or 30.

This leads us to several conclusions. First, if prior studies have grossly underestimated the cost of aneuploidy from a consumer viewpoint, and particularly since having a specific cutoff age has negative consequences, then amniocentesis might best be provided at all ages, although as Hook and Chambers (23) have suggested, the individual might be asked to pay a higher share of the cost at lower ages. It should be noted that an imputed negative worth of a case of Down syndrome of even 2 or 3 times Andreano and McCollum's estimate (1) (much less one 20 times that estimate) would be sufficient to justify amniocentesis at all ages, even at the one in 2,000 cases probability that is Hook's minimum estimate for age 21 yr.

Second, if the burden of aneuploidy has such a high value, then the costs of environmental factors that raised rates, even if only slightly, would be high. Even if it turned out that only the 3% of cases were involved, aggregate costs or benefits could be substantial. This in turn suggests the value of further research on possible environmental effects, both through laboratory experimentation and through truly multivariate epidemiological studies. Even if there is still uncertainty about results, the potential payoffs justify the attempt.

CONCLUSIONS

These results, like cost-benefit analysis itself, are dependent on existing social conditions and attitudes. If there were different social attitudes toward Down syndrome and other birth defects, and if labor market and career options for women were different, then the costs we have measured would also be different. The costs of Down syndrome and other conditions associated with aneuploidy are not biological givens. In many cases of retardation, the choice between institutionalization and outpatient care, and the degree to which the individual can be put to socially useful work, are determined by social arrangements and attitudes.

There has been increased awareness in recent years that evaluation of retardation is more problematic than previously supposed (e.g., Ref. 32). Many of the severely handicapped are in fact capable of much more than initially assumed. There is reason to believe that many of the disabled could under certain conditions become happily integrated and productive members of society. The problem may not be that the individual is not capable of meaningful work, but that the labor market is increasingly structured to demand credentials far beyond the necessity demanded by the job. A great many types of work would be well suited to Down syndrome and other disabled individuals. But while there is a large surplus of labor, it is unlikely that those "handicapped" will be allowed to demand their "fair" inclusion. Thus the figures that Conley (8) and others have estimated for the reduced earnings of the "retarded" reflect not only the biological possibilities, but the short-term state of our social prejudices.

This an issue that goes beyond economic studies, for there is no evidence to indicate that disability necessarily "retards" the individual's emotional abilities. A Down syndrome child is as capable of "hurt" as an intellectually gifted child.

The measured costs of aneuploidy and the still unmeasured costs of stigma for mothers who provide "less than perfect" children reflect social attitudes in yet another way. The stigma reflects a value we place on success, particularly in work, or on the creation of career-capable offspring. We frequently define ourselves by our "work," generally paid employment, and we gain status from our position in the labor market. The values we place both on mothers' stigmas and on Down syndrome children's work reflect this form of status. It is one of those givens that cost-benefit analysis takes as a condition for its calculations, but it is one that might be considered as a candidate for eventual change.

There has, of course, been challenge in recent years of the assumptions of need for institutionalization. There has been a growing awareness that longterm institutionalization is morally problematic and extremely expensive. But until better social services are provided in a community setting, costs are likely to be borne either by large institutional warehousing facilities or by families. The conditions taken by the existing economic studies are probably realistic in this regard, as far as they go, but it is important to realize those costs could change with the advent of even rather mild social reforms. Klarman's concept of the "obvious, partial remedy" would seem to be relevant here.

In the absence of these reforms, we are left with a world in which costs of care and institutionalization are high, and in which labor market and stigma effects are serious. And economic analysis is based on the measurement of policies in the world as it is. For this existing situation, our exercise in analysis has brought us to several conclusions, which we can summarize here.

We conclude that despite their problems, economic studies have demonstrated under a variety of possible assumptions that aneuploid conditions (Down syndrome being the most studied) have high social costs. Greater precision in the reporting and dissemination of epidemiological information is important, to avoid some of the unnecessary labor market costs that stem from the attempt to avoid risk. There is also a need for more truly multivariate and longitudinal epidemiological studies to sort out just what the observed maternal-age effect is an index for, and to provide leads to possible environmental factors.

Finally, research on possible environmental impacts on aneuploidy and other genetic conditions should be supported. Despite uncertainty of outcome, potential benefits to any positive results are so substantial that the search should be undertaken.

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MEIOTIC INVESTIGATIONS OF ANEUPLOIDY IN THE HUMAN

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INTRODUCTION

It is widely recognized that roughly 50% of all first trimester miscarriages are chromosomally abnormal and that the birth of an aneuploid child presents a heavy burden to parents and to society. The aneuploid condition is generally attributed to a nondisjunctional event at one of the two meiotic divisions which take place during oogenesis and spermatogenesis. Attention is largely focused on the trisomy 21 condition--the most common numerical chromosomal abnormality at birth. In a compilation of many studies into the parental origin of the extra chromosome 21 in individuals with Down syndrome assigned by Q-band polymorphism, Juberg and Mowrey (24) found that 64% of cases were attributed to nondisjunction at the first meiotic division during oogenesis, 16% to nondisjunction at the second meiotic division during oogenesis, 12% to nondisjunction at the first meiotic division during spermatogenesis, and 8% to nondisjunction at the second meiotic division during spermatogenesis.

Direct estimates of the rates of nondisjunction at these divisions can be made only by comparison of the level of aneuploidy in cells approaching the division with the level in those having completed the division in question. Thus, observation of oocytes and spermatocytes at first prophase and at diakinesis/first metaphase provides information about the rate of nondisjunction during premeiotic or gonial divisions; observation of cells at second metaphase indicates the combined gonial and first meiotic rates of nondisjunction; and investigation of spermatozoa (as is separately described by Martin, this Volume) provides information on the combined gonial, first and second meiotic rates of nondisjunction in the male.

Although aneuploidy is a condition in which a cell contains more or fewer chromosomes than an exact multiple of the haploid number, the absence of chromosomes in preparations might be an artifact of the technique and therefore, by convention, only supernumerary chromosomes are considered as evidence for nondisjunction. This chapter reviews the information available on nondisjunction in the human from observations of meiotic preparations obtained from ovarian and testicular biopsies.

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TECHNIQUES

Air-Drying and Sequential Chromosome Staining

The air-drying technique based on Evans et al. (5) is suitable for analysis of meiotic preparations from the adult testis and the fetal ovary by light microscopy. Material from testicular biopsies must be processed immediately as spermatogenic epithelium degeneration is rapid. However, fetal ovaries may be left in fixative (3:1 methanol/acetic acid) for about 12 hr and a further delay of up to 72 hr may not be disadvantageous. Cells are spread from fixative by dropping onto cold (4°C) glass slides which are left to dry at room temperature.

The application of a sequential staining procedure allows unambiguous chromosome counts to be made and permits the karyotyping of the majority of spermatocytes at first and second metaphase. A triple-staining technique is described by Laurie et al. (25) (modified from Laurie et al. [26]) which combines quinacrine mustard fluorescence, lacto-propionic orcein staining, and C-banding. The technique may be extended further by incorporating Distamycin A/DAPI fluorescence after Q-banding to produce a quadruple-staining method (42).

Surface-Spreading

Conventional air-drying techniques do not provide adequately spread cells at late zygotene and pachytene for quantitative analysis, but this may be achieved by surface-spreading which allows visualization of the synaptonemal complexes, i.e., the structures formed between homologous chromosomes at these stages. Spreading may be performed directly on a microscope slide using sucrose or detergent as the spreading solution. Analysis can be performed on silver-stained preparations by light microscopy and on preparations stained with ethanolic phosphotungstic acid (PTA) by electron microscopy. Details of the surface-spreading methods we use for fetal oocytes are given by Wallace and Hulten (51), and for spermatocytes by Saadallah and Hulten (43). These are modifications of the method described in Jones et al. (23).

Chromosomes are identified in surface-spread preparations mainly by ranking according to length. Centromere positions (when discernible) and nucleolus associations assist identification. In our experience, G-, R-, Q-, and C-banding techniques are not effective in these preparations. There is, however, a possibility of identifying centromeres specifically using an immunofluorescent technique which has been successfully applied to the synaptonemal complexes of human spermatocytes (Moses, pers. comm.).

FEMALE MEIOSIS

Staging of Female Meiosis

Germline cells in the female fetus mature rapidly into oogonia and, after a finite number of mitotic divisions, transform into oocytes which enter first meiotic prophase from around the ninth week of fetal gestational age. Leptotene and zygotene are thought to be brief compared to pachytene which lasts about 2 weeks and may be observed in fetuses from the 11th week, although it is predominant at the fetal age of 18-22 weeks. Oocytes reach the diplotene stage from the 13th week of gestational age and all have done so by the time of birth. Oocytes remain at diplotene until ovulation: the first and second meiotic divisions in vivo do not occur until ovulation and fertilization, respectively.

Pachytene investigations have been made from traditionally air-dried preparations and from surface-spread preparations of fetal ovarian material. Some first and second metaphase investigations have been made by short-term in vitro culture of oocytes from adult ovarian biopsies (see Fig. 5 and 6), but to date no analyses of the in vivo situation have taken place because of the inherent problems in obtaining human oocytes at the required stages.

Pachytene Analysis of Oocytes

Traditional techniques. A number of investigations have been made into chromomere mapping of human pachytene chromosomes in the oocyte by air-drying techniques. Early investigations (29,30) were restricted to certain chromosomes because cells were not sufficiently well-spread to permit analysis of the complete chromosome complement. Later investigations (6,21) with improved hypotonic swelling allowed analysis of the complete chromosome complement of many cells. In the single study using traditional techniques (6), among 1,050 oocytes analyzed from 35 fetuses with a normal karyotype, none was reported to be numerically abnormal. The relevance of this study for estimation of premeiotic nondisjunction is unclear, however, as the method used to select analyzed cells was not indicated.

Surface-spreading. Although the surface-spreading technique has rapidly emerged over the last few years as a method for visualizing synaptonemal complexes it has only recently been extended to human oocytes. The first reports using this method in the normal fetus were from Sung et al. (47) and Hulten et al. (12). A small number (15) of human pachytene oocytes from 3 normal fetuses were observed by Sung et al. (47) in EM studies of preparations stained with ethanolic PTA. All were numerically normal. The method used to select analyzed cells, however, was not indicated. The report of Hulten et al. (12) included preliminary observations of 16 oocytes from one normal fetus. Each cell contained 23 bivalents. A more comprehensive study has now been completed (51) including observations of 112 pachytene oocytes from 2 control fetuses. Cells were selected strictly on the basis of the quality of the spread (Fig. 1-4). One cell contained a small supernumerary chromosome interpreted as a univalent 21 (Fig. 2). Chromosome breakage was not common and appeared to be a preparation artifact (Fig. 3). A recent study by Speed (45), specifically intended to detect abnormalities, included analyses of 154 pachytene oocytes from 9 fetuses by light microscopy and 162 from 6 fetuses by electron microscopy. No supernumerary chromosomes were observed.

Thus, observation of a total of 459 surface-spread pachytene oocytes included only one with a supernumerary chromosome (Tab. 1). This suggests that the frequency of nondisjunction in oogonia giving rise to supernumeracy may be as low as about 0.2%. It is perhaps interesting to note that the extra chromosome observed was interpreted as number 21.

One pair of univalents was detected by Wallace and Hulten (51) in 1/112 oocytes (Fig. 4). Speed (45) observed univalents in 2/154 oocytes by light microscopy and in 17/162 oocytes by electron microscopy (Tab. 2). Univalence may be caused by late pairing, by desynapsis, or it may be the result of failure of pairing (asynapsis). If univalents segregate randomly at first anaphase, nondisjunction at the first meiotic division may result. The contribution of this event to aneuploidy in human fetuses is uncertain.

First and Second Metaphase Analysis of Oocytes

Jagiello and Lin (22), using the in vitro culture technique of Tarkowski (48), made observations of over 174 human oocytes (Tab. 1). No disjunctional abnormalities were seen, and thus there was no indication of

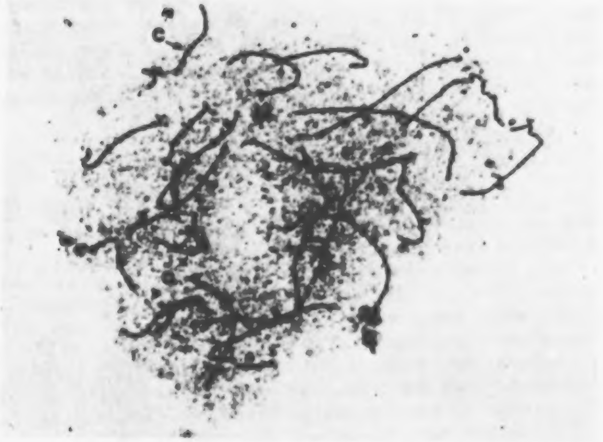

Fig. 1. Pachytene oocyte from a chromosomally normal anencephalic fetus. The 23 bivalents are fully paired. Some centromeres (C) are visible.

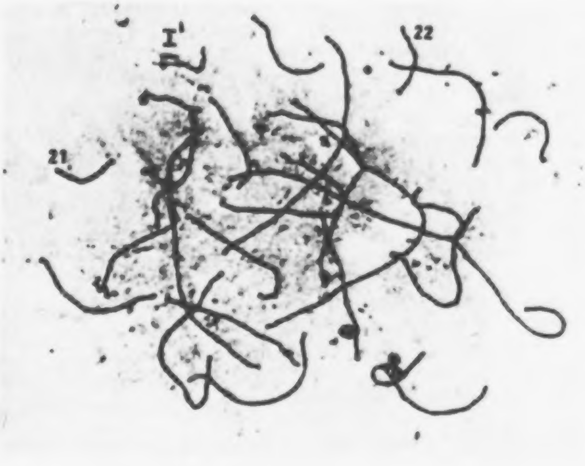

Fig. 2. Pachytene oocyte from a phenotypically normal fetus with an extra chromosome interpreted as a univalent 21 (I).

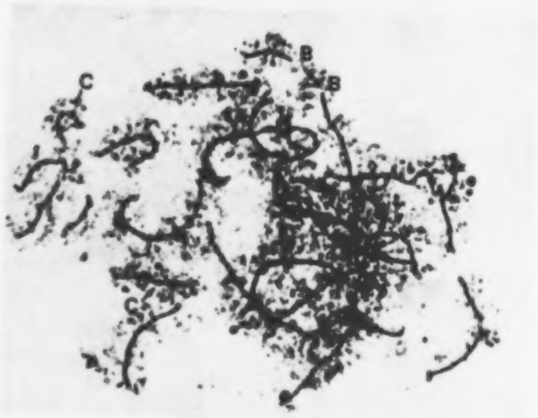

Fig. 3. Pachytene nucleus from an anencephalic fetus with 23 bivalents. The longest bivalent has 2 breaks (C = centromere and B = break).

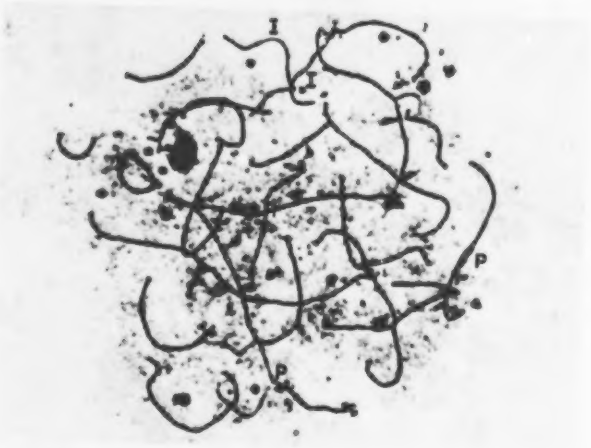

Fig. 4. Pachytene oocyte from a phenotypically normal fetus. One middle-sized pair of homologs remains unsynapsed, while 2 others show partial pairing (I = univalent and P = partially paired bivalent).

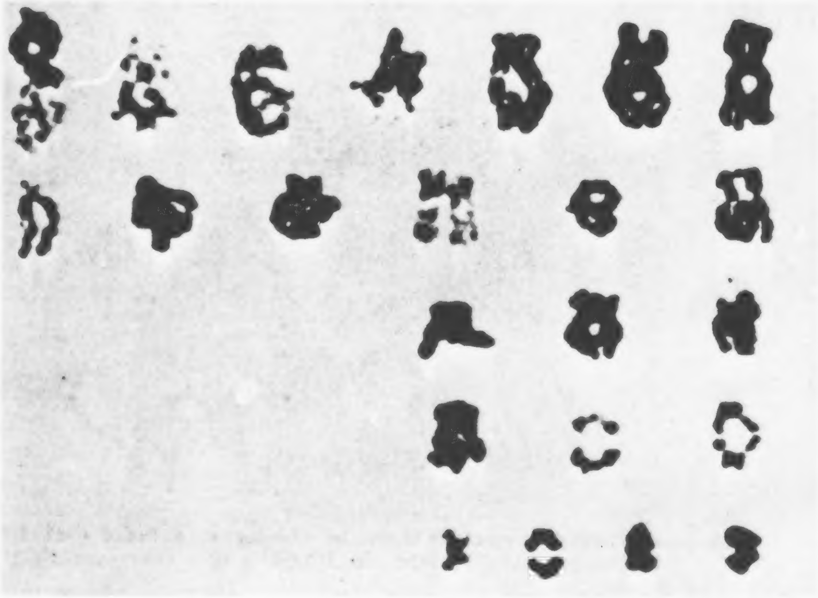

Fig. 5. First metaphase oocyte with 23 bivalents arranged according to size (from Yunchen [52]).

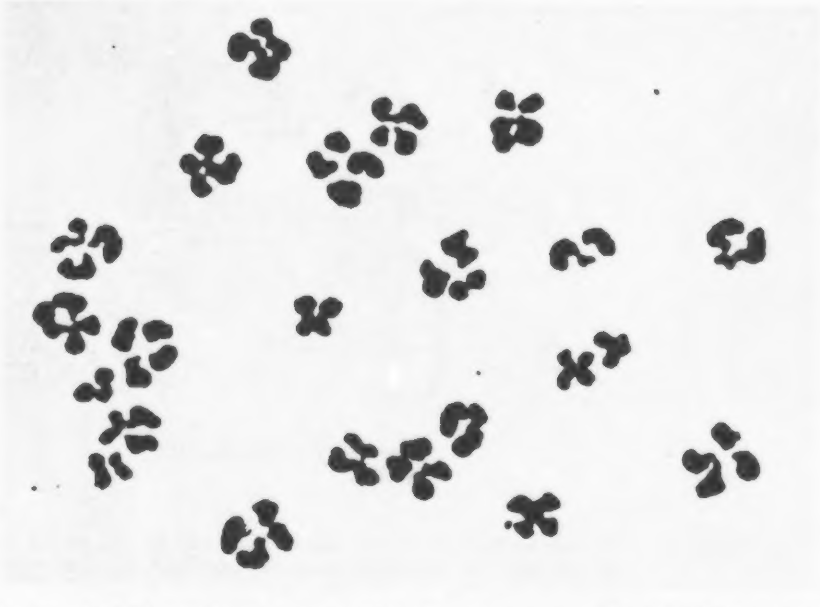

Fig. 6. Second metaphase oocyte with 23 chromosomes (from Jagiello et al. [19]).

Tab. 1. Assessment of premeiotic nondisjunction in the female.

	Number of subjects/ fetal ovaries	Number of oocytes	Supernumeracy (%)
Pachytene			
Surface-spreading			
Sung et al. (47)	3*	15*	0 (0)
Hulten et al. (12)	1	16	0 (0)
Speed (45)	9	154	0 (0)
	6*	162*	0 (0)
Wallace and Hulten (51)	2	112	1 (0.9)
	21	459	1 (0.2)

	Number of oocytes	Supernumeracy (%)
First metaphase		
In vitro culture of oocytes from adult ovarian biopsy		
Jagiello and Lin (22)	174	0 (0)

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* EM

oogonial nondisjunction. In observations of 411 second metaphase oocytes (19; see Fig. 6), 6 contained 24 bodies (Tab. 2); however, it was suggested that these were probably due to separations of chromatids at the centromere. This problem could be the result of cells entering early second anaphase and can be overcome by C-banding which identifies centromeres and allows accurate chromosome counts to be made. Without further investigations using this technique it is not possible to draw any conclusions on first meiotic female nondisjunction.

MALE MEIOSIS

Staging of Male Meiosis

Germline cells in the human male develop during childhood into spermatogonial stem cells. At puberty these cells begin to proliferate by repeated mitotic divisions and mature primary spermatocytes are formed after about 1 month. Spermatocytes enter first meiotic prophase with a preleptotene stage lasting approximately 1 day.

Leptotene and zygotene occupy 1 week and are followed by pachytene with a duration of approximately 2 weeks. The remaining first meiotic stages and all the second meiotic stages are completed within 1 day. The resultant spermatids mature into motile and fertile spermatozoa through spermiogenesis—a complex transformation without further cell divisions lasting about 3 weeks.

All meiotic stages are present in the adult human testis and thus all stages can be investigated in preparations of testicular biopsy material.

Tab. 2. Assessment of first meiotic nondisjunction in the female.

	Number of subjects/ fetal ovaries	Number of oocytes	Univalence (%)
Pachytene			
Surface-spreading			
Sung et al. (47)	3*	15*	0 (0)
Hulten et al. (12)	1	16	0 (0)
Speed (45)	9	154	2 (1.3)
	6*	162*	17 (10.5)
Wallace and Hulten (51)	2	112	1 (0.9)
	21	459	20 (4.4)

	Number of oocytes	Supernumeracy (%)
Second metaphase		
In vitro culture of oocytes from adult ovarian biopsy		
Jagiello et al. (19)	411	0** (0)

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* EM

** Six cells contained 24 structures interpreted as containing separated chromatids.

Testicular biopsies for meiotic chromosome analysis can generally be obtained from 3 categories of patients: (a) from infertile men where an extra piece of material is taken in addition to that for histological diagnostic purposes; (b) from men with mitotic chromosome abnormalities who volunteer a donation for research purposes; and (c) from individuals who volunteer a donation while undergoing therapeutic testicular surgery (e.g., for vasectomy, inguinal hernia, or prostatic cancer).

Analysis of Spermatogonia

Premeiotic spermatogonial metaphase is a difficult stage to analyze due to the poorly spread nature of the chromosomes in air-dried preparations and the presence of ambiguous secondary constrictions in chromosomes 1 and 9. Thus, no quantitative studies of chromosomes at this stage have been performed. On a historical note it may be added that long before the correct diploid chromosome number in humans was finally established as 46 (8,50), Painter (35) reported that in spermatogonial mitosis "the counts range from 45 to 48 apparent chromosomes, although in the clearest equatorial plates so far studied only 46 chromosomes have been found." Two years later, however, he concluded that the correct diploid number was 48 (36), and we are wondering if he could have been fooled by the extended secondary constrictions of chromosomes 1 and 9 (e.g., Fig. 3 [14] and Fig. 3b [15]).

Pachytene Analysis of Spermatocytes

Traditional techniques. A number of studies have been made of chromomere mapping of human pachytene spermatocytes using air-drying techniques. Hungerford (18) described accurately countable pachytene complements, but numerical abnormalities were not reported, nor was the number of spermatocytes observed indicated. Two more recent studies using similar techniques, by Jagiello and Fang (20) and by Luciani et al. (31), analyzed over 130 spermatocytes from 5 subjects, and 120 spermatocytes from 12 subjects, respectively. No numerical abnormalities were reported in either investigation. The method used to select analyzed cells, however, is unclear, so the relevance of this information on the rate of spermatogonial nondisjunction is uncertain.

Three-dimensional reconstruction. Holm and Rassmussen (10,11) have analyzed the synaptonemal complexes in 22 spermatocytes from 5 subjects using 3-dimensional reconstruction of serial EM sections. None of these cells contained an extra chromosome.

Surface-spreading. Surface-spreading, initially applied to Locusta spermatocytes (4), was first extended to human spermatocytes by Moses et al. (33). Fletcher (7) subsequently described a silver-staining method to permit analysis by light microscopy, and Navarro et al. (34) described a method for sequential study of synaptonemal complexes by light and electron microscopy.

Despite the study of many pachytene spermatocytes, no aneuploidy has been observed (Tab. 3). Solari (44) karyotyped 22 selected pachytene spermatocytes from 3 individuals and all were numerically normal. In a study including observations of 44 cells from 3 men (12), no cell was numerically abnormal. Saadallah (41), in observations of 1,073 cells (see Fig. 7), saw no hyperdiploidy.

Analysis of Spermatocytes at Diakinesis/First Metaphase

Analysis of spermatocytes at diakinesis/first metaphase provides additional information about the rate of premeiotic nondisjunction to that obtained by analysis of spermatocytes at pachytene. Detection of supernumerary chromosomes at diakinesis/first metaphase should be straightforward, even using conventional staining techniques. Although many thousand cells from normal subjects have been analyzed (e.g., Ref. 15, 16, and 17;

Tab. 3. Assessment of premeiotic nondisjunction in the male.

	Number of subjects/ testicular biopsies	Number of spermatocytes	Supernumeracy (%)
Pachytene			
Surface-spreading			
Solari (44)	3	22*	0 (0)
Hulten et al. (12)	3	44**	0 (0)
Saadallah (41)	8	1,073**	0 (0)
	14	1,139	0 (0)

	Number of spermatocytes	Supernumeracy (%)
Diakinesis/first metaphase		
Air-drying		
Various authors	Many thousand	0 (0)

* Selection unknown.
** Selected for clarity.

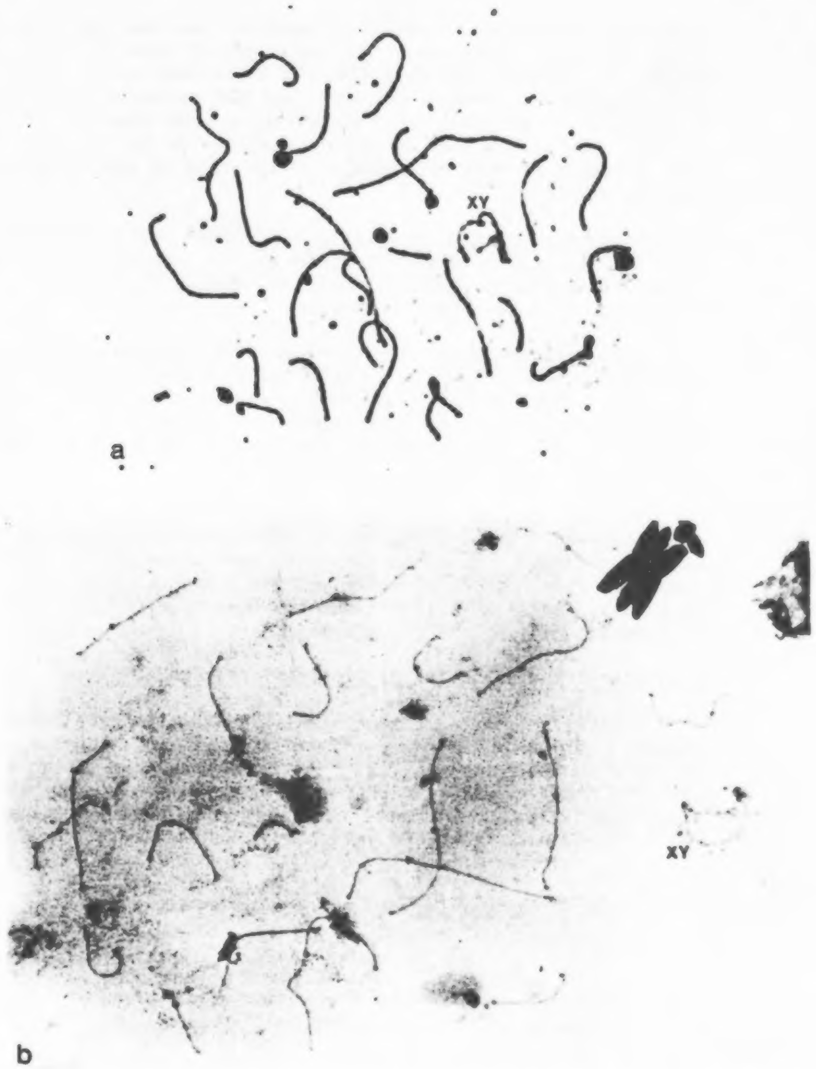

Fig. 7. Pachytene spermatocytes with 23 bivalents from a control with a 46,XY mitotic karyotype. (a) Light microscopy. (b) Electron microscopy.

see Fig. 8), to our knowledge none contained an extra chromosome (Tab. 3). These observations reinforce the suggestion, from analysis of pachytene spermatocytes, that the rate of spermatogonial nondisjunction seems to be very low.

Analysis of Spermatocytes at Second Metaphase

The only relevant investigation to date, a recent pilot study of 200 second metaphase spermatocytes (25), was performed by applying a sequen-

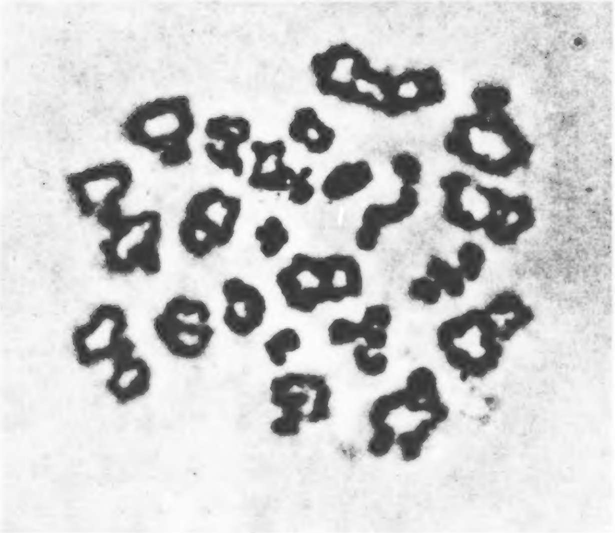

Fig. 8. Spermatocyte at diakinesis/first metaphase with 23 bivalents from a control with a 46,XY mitotic karyotype.

tial-staining regime to air-dried chromosome preparations (see Fig. 9). The purpose of the study was to obtain a direct estimate for the level of aneuploidy, however, of the 200 cells analyzed from 6 men with apparently normal karyotypes, none was hyperhaploid (the frequency of hypohaploidy was 10%, and the frequency of cells with breakages, i.e. "hyperhaploid" with acentric fragments, was 5%; see Tab. 4). This result is not inconsistent with expectations of first meiotic nondisjunction derived from other lines of investigation. Taking the frequency of hyperhaploidy in spermatozoa as 2.4% (32) and assuming that first meiotic nondisjunction accounts for 60% of this (from studies of chromosome 21 polymorphism segregation [24]), then 1.44% of second metaphase spermatocytes would be hyperhaploid. This is not significantly different from an observation of zero in a sample of 200 cells. Clearly an enlarged study is required to provide a more precise estimate of the level of hyperhaploidy in second metaphase spermatocytes and to perform a more accurate test of departure from the expected value.

Analysis of Spermatozoa

Data from investigations of spermatozoa provide information on the combined rates of spermatogonial, first meiotic and second meiotic nondisjunction in the human male. Information obtained from the analysis of spermatozoa is presented in detail elsewhere (Martin, this Volume).

Two types of investigation have been made. First there is the study of specific chromosomes using special staining techniques to identify "double bodies." This approach has been applied to chromosome 1 (9), chromosome 9 (e.g., Ref. 1), and the Y chromosome (e.g., Ref. 38 and 46). Estimates of the frequency of "double bodies" for these chromosomes are between 1 and 2%, which seem to be high when it is considered that if all chromosomes behaved in the same way the overall level of aneuploidy in human spermatozoa would be around 40%.

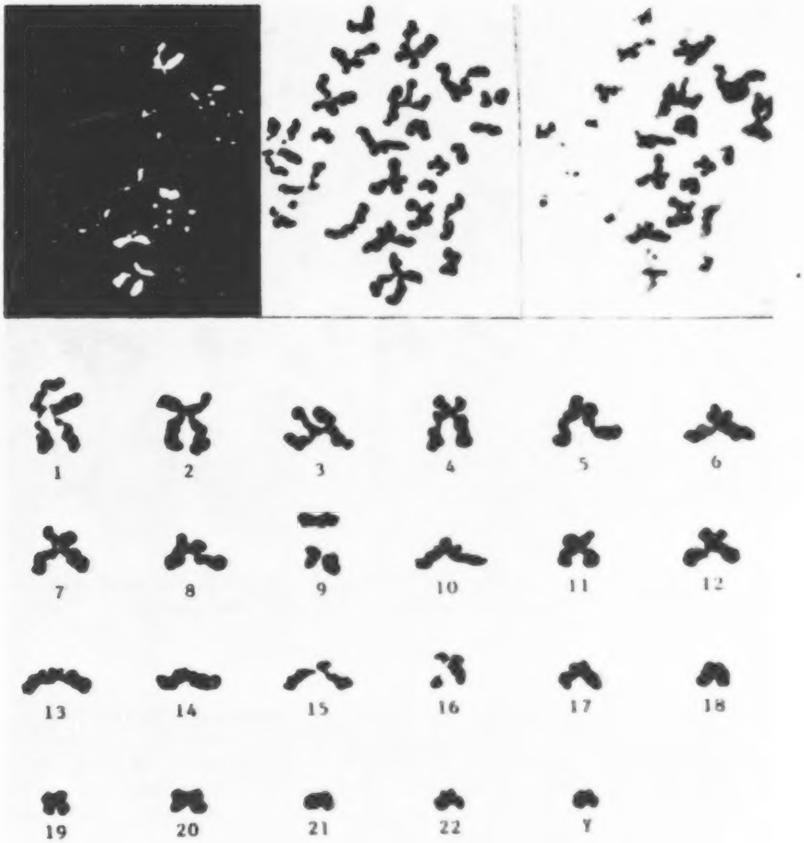

Fig. 9. Karyotype and triple-staining of a second metaphase spermatocyte from a control with a 46,XY mitotic karyotype (from Laurie et al. [25]).

The second type of investigation, first reported by Rudak et al. (40), has involved the analysis of the complete chromosome complement following the in vitro fusion of human spermatozoa with Syrian hamster eggs. The analysis of 1,000 spermatozoa by Martin et al. (32) has produced estimates of 5.2% aneuploidy and 2.4% hyperhaploidy (24/1,000 cells). In another more recent study (3) the mean frequency of hyperhaploid spermatozoa was only 0.7% (17/2,468 cells). Despite these differences, the levels of aneuploidy estimated by this method are surely more accurate indications of the combined rates of spermatogonial, first meiotic and second meiotic nondisjunction than those obtained by "double body" analysis.

SUMMARY AND DISCUSSION

The incidence of aneuploidy is higher in the human than in any other organism investigated. It is generally thought that aneuploidy is caused by a nondisjunctional event at first or second meiosis during gametogene-

Tab. 4. Assessment of first meiotic nondisjunction in the male.

	Number of subjects/testicular biopsies	Number of spermatocytes	Hypohaploidy (%)	"Hyperhaploidy" with acentric fragment(s) (%)	True hyperhaploidy (%)
Second metaphase					
Air-drying					
Laurie et al. (25)	6	200	20 (10)	10 (5)	0 (0)

sis, but surprisingly few investigations have been performed on meiotic preparations to verify this directly. Since we last reviewed the possibilities of detecting chromosome abnormalities in the germline (16), improved techniques have been developed and are now readily available for adequately assessing first meiotic nondisjunction in the human by direct observation of preparations from ovarian and testicular biopsies (12,25). The main problem remaining is the accessibility of this material.

We can be reasonably sure from chromosome counts at first meiosis that the level of premeiotic nondisjunction in the human male is very low, and there is no indication yet that it is much higher in the female. Chromosome counts at second meiosis are, therefore, likely to provide a relevant assessment of first meiotic nondisjunction. As yet, however, the number of appropriate counts performed at second meiosis is too small to draw any definite conclusions about first meiotic nondisjunction in either sex. All that can be said at the moment is that in the male there is no significant deviation in the frequency of aneuploidy from expectations derived from investigations of numerical aberrations in spermatozoa.

Aneuploidy as a general subject has been admirably reviewed by Bond and Chandley (2). It is difficult to speculate as to which specific mechanisms cause the raised levels of meiotic nondisjunction and lead to the high incidence of aneuploidy seen in the human. No specific investigations concerning aneuploidy have been performed regarding the known predisposing factors such as maternal age and ionizing radiation. There are some indications that physiological rather than chronological age is of importance and interest has recently focused on age-related spindle disturbance (Chandley, pers. comm.).

There are also some indications that parents of either sex with structural chromosome aberrations may be predisposed to bearing trisomic offspring (e.g., Ref. 28). The meiotic analysis of structural chromosome abnormalities has not revealed any detectable relevant disturbance of first meiotic chromosome pairing in male carriers (Hulten and Saadallah, unpubl. observ.), but we have recently demonstrated changes in the chiasma pattern at diakinesis/first metaphase of the structurally abnormal chromosomes, and also of the other chromosomes (27,37). It would be of further interest to investigate the meiotic chromosome pairing and chiasma pattern of karyotypically normal parents who have had trisomic offspring. This is only feasible if fathers are prepared to volunteer a testicular biopsy for research purposes. It is possible that some such men could be meiotic mutants with abnormal first meiotic synapsis and random chromosomal segregation at first anaphase, or some could have some other disturbance in chiasma formation (see Ref. 13, 39, and 49), such as a low chiasma frequency which is occasionally found in infertile patients.

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DISCUSSION

HOOK: Would you comment on the apparent difference between Drs. Martin's and Brandriff's studies on sperm (see Martin, this Volume) and your study with regard to the apparent absence of evidence for supernumeraries in your study of testicular material and the higher rates of numerical abnormalities in the sperm?

HULTEN: I do not think there is a difference. We have only looked at 200 cells after second metaphase--that is the first nondisjunction evaluated--and if we extrapolate from the investigations on trisomic Down syndrome and the mitotic marker investigations, then we would not expect more than about 3 or perhaps 4 sperm to be numerically abnormal in such a small sample. Therefore, I think that the only thing we can do is to extend the study.

FORD: Dr. Hulten had emphasized the possibility of the crossovers in the small chromosomes actually giving incorrect information about where the error occurred. That may suggest that we are really looking for a meiosis 2 error, whereas if we are not looking for a pairing error, but rather a segregation error, then the data from such studies could still be correct.

HULTEN: Most of the material on meiotic investigations is concerned with numerical and structural abnormalities in males. In translocation heterozygotes there does seem to be a change in the chiasma distribution, both within the chromosomes involved in the translocation and also in other chromosomes. So there is an interchromosomal effect.

CHROMOSOMAL ABNORMALITIES IN HUMAN SPERM

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SUMMARY

The ability to analyze human sperm chromosome complements after penetration of zona pellucida-free hamster eggs provides the first opportunity to study the frequency and type of chromosomal abnormalities in human gametes. Two large-scale studies have provided information on normal men. We have studied 1,426 sperm complements from 45 normal men and found an abnormality rate of 8.9%. Brandriff et al. (5) found 8.1% abnormal complements in 909 sperm from 4 men. The distribution of numerical and structural abnormalities was markedly dissimilar in the 2 studies. The frequency of aneuploidy was 5% in our sample and only 1.6% in Brandriff's, perhaps reflecting individual variability among donors. The frequency of 24,YY sperm was low: 0/1,426 and 1/909. This suggests that the estimates of nondisjunction based on fluorescent Y body data (1% to 5%) are not accurate.

We have also studied men at increased risk of sperm chromosomal abnormalities. The frequency of chromosomally unbalanced sperm in 6 men heterozygous for structural abnormalities varied dramatically: 77% for t11;22, 32% for t6;14, 19% for t5;18, 13% for t14;21, and 0% for inv 3 and 7. We have also studied 13 cancer patients before and after radiotherapy and demonstrated a significant dose-dependent increase of sperm chromosome abnormalities (numerical and structural) 36 months after radiation treatment.

INTRODUCTION

Humans have a very high frequency of meiotic chromosomal abnormalities and this results in pregnancy loss and the birth of abnormal children. There have been many studies, in humans and experimental animals, to try to elucidate the causes of meiotic chromosomal abnormalities and to identify factors which increase the frequency of these abnormalities. In the past, these studies have been hampered by the inability to directly analyze the chromosomes in human gametes. An operation is required to obtain human eggs, and chromosomes are not visible in human sperm until pronuclei are formed following fertilization.

In 1978, a significant advance occurred in meiotic research when Rudak et al. (23) reported a new technique which allows direct analysis of the chromosome constitution of human sperm. They fertilized zona pellucida-free golden hamster eggs with human sperm and analyzed the chromosomes of the male pronucleus in the fertilized egg. Since then, my laboratory has studied the frequency and type of chromosomal abnormalities in sperm from normal healthy men (15,17), men heterozygous for structural chromosomal rearrangements (2,3,4,16,18) and cancer patients exposed to radiotherapy (20). These various studies will be discussed and our results on sperm chromosomal abnormalities in normal men will be compared to those of Brandriff et al. (5).

MATERIALS AND METHODS

A detailed description of the technique for human sperm chromosomal analysis has been provided elsewhere (19). The following methods provide an outline of the technique.

Sperm Processing

Semen samples are obtained at the laboratory. Traditional semen analysis is performed on an aliquot of the sample. The sperm is washed in a modified Krebs-Ringer solution called BWW, by 3 centrifugations at 600 g. The sperm are resuspended in BWW for a final concentration of 10^7 sperm/ml. Droplets (each 0.2 ml) of the sperm solution are placed under paraffin oil in sterile Falcon petrie dishes and incubated at 37°C in 5% CO_2 in air for 5 hr to allow for capacitation.

Egg Processing

Female golden hamsters are superovulated and the oocytes are dissected from the Fallopian tubes. The cumulus mass is dispersed with 0.1% hyaluronidase and the zona pellucida is dissolved with 0.1% trypsin. The oocytes are washed in BWW and placed into the drops containing the capacitated sperm. They are incubated for 3 hr at 37°C in 5% CO_2 in air to allow sperm penetration of hamster oocytes to occur.

Fertilization Check

Approximately 40 oocytes are checked for evidence of sperm penetration after 2 hr incubation. Squash mounts are prepared to detect swollen sperm heads (and tails) and/or male and female pronuclei.

Culture of Fertilized Eggs

After a 3 hr incubation, the oocytes are washed in medium F10 to remove excess sperm and transferred to 0.2 ml drops of F10 under paraffin oil for 12.0 to 12.5 hr incubation. The next morning the oocytes are transferred to 0.2 ml drops of F10 containing 0.4 μg/ml Colcemid under paraffin oil and incubated for a further 4 to 7 hr.

Slide Preparation

The oocytes are then transferred to hypotonic solution (1% sodium citrate) for 3 to 6 min. The oocytes are fixed on slides by dropping four 20 μl drops of fixative (3:1 95% ethanol:glacial acetic acid) on them from a height of approximately 1 cm. After 2 weeks chromosomes are stained with 0.5% quinacrine dihydrochloride (pH 4.5) for 15 min followed by 3 rinses in distilled water (ph 4.5) for a total of 10 min. The chromosomes are photographed with a Zeiss fluorescent microscope.

RESULTS AND DISCUSSION

Analysis of Sperm Complements From Normal Men

Sperm donors. The donors were healthy men with no history of radiotherapy or chemotherapy. We have studied 45 men, 28 of proven fertility and 17 of unproven fertility. The range in age was 23 to 50 years. Brandriff et al. (5) studied 4 men, aged 34 to 39 years, of whom 3 were of proven fertility.

Level of sperm chromosome identification. This technique provides sperm chromosome complements in which the individual chromosomes can generally be identified by their quinacrine banding patterns. However, in approximately 10% of the spreads, one or more chromosomes can be identified only to the Denver group classification (A through G). A chromosome spread of a normal 23,X sperm complement is shown in Fig. 1.

Extended heterochromatic regions. The gap seen in the centromeric region of many of the chromosomes in Fig. 1 and 2 is a common finding in all laboratories that have had success with this technique. The "gaps" are actually areas of reduced chromatin coiling and a chromatin strand connecting the 2 chromosome arms can be seen using scanning electron microscopy (unpubl. observ.). The chromatin despiralization is most evident in the heterochromatic regions of chromosomes 1, 9, 16, and Y. The apparent decreased condensation of the Y is reflected in a duller fluorescence than is normally seen for this region in other tissues. The centromeric chromatin uncoiling is present in the hamster pronuclear chromosomes as well as the human chromosomes and this phenomenon is not restricted to the interspecific human-hamster cross. Centromeric chromatin uncoiling has also been observed in the pronuclear chromosomes of bovine zygotes after in vivo

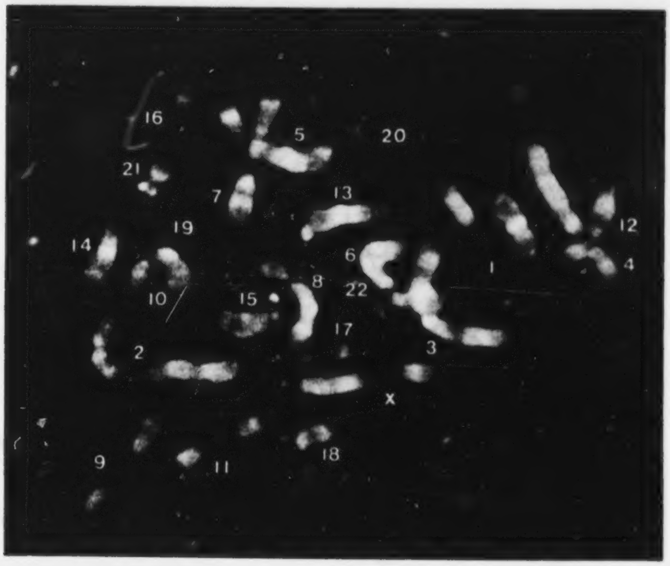

Fig. 1. A Q-banded chromosome spread of a normal 23,X human sperm complement (from Ref. 19).

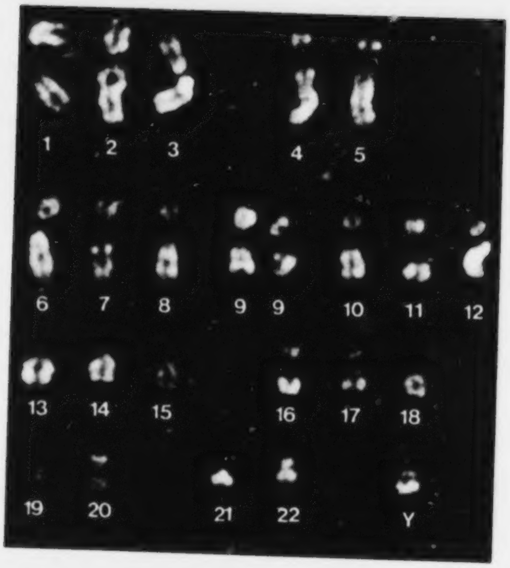

Fig. 2. A Q-banded karyotype of a 24,Y + 9 human sperm complement (from Ref. 17).

fertilization (Allan King, per. comm.) and in the pronuclear chromosomes of human zygotes after in vitro fertilization (24). The significance of this uncoiling of the heterochromatin is unknown. It is possible that it may be an artifact or it may have some role in embryonic development and the phenomenon deserves more study.

Frequency and type of sperm chromosomal abnormalities. Rudak et al. (23) analyzed 60 sperm from one male. Thirty-one were 23,X, 26 were 23,Y, and 3 were aneuploid. Therefore, the frequency of aneuploidy for this small sample was 5%.

We first reported on 240 sperm chromosome complements from 18 normal men (15), and then extended this sample to 1,000 complements from 33 normal men (17). In this report we will discuss our results on 1,426 sperm chromosome complements from 45 normal men. We found an abnormality rate of 8.9% in this sample. Brandriff et al. (5) studied 909 sperm complements from 4 normal men and found 8.1% to be abnormal. A summary of these 2 studies is presented in Tab. 1. An example of an abnormal sperm chromosomal complement is presented in Fig. 2. In our study, numerical abnormalities (5%) were more common than structural abnormalities (3.9%) as they are in studies of spontaneous abortions (11) and newborns (12). The opposite was true in the study of Brandriff et al.: only 1.6% of the abnormalities were numerical and 6.5% were structural. The most likely explanation for these disparate results is individual variability among the donors. Brandriff et al. (5) found significant differences in the individual frequencies of structural abnormalities among their 4 donors (range 1.3% to 10.4%). And this was confirmed in an expanded study on 11 men, presented at the American Society of Human Genetics meetings in 1984 (6): the range in the frequency of structural abnormalities was 1.9% to 15.1%. The range in the frequency of abnormalities in our study was also large (0% to 28%). Two men were outliers with 23% and 28% abnormalities, respectively, of which more than 80% were structural rather than numerical abnormalities.

Tab. 1. Summary of sperm chromosomal abnormalities in two large studies.

	Martin et al.	Brandriff et al.
Overall abnormality rate	8.9%	8.1%
Aneuploid	5.0%	1.6%
Hyperhaploid	1.8%	0.6%
Hypohaploid	3.1%	1.0%
Double aneuploidy	0.1%	--
Structural	3.9%	6.5%
Chromosome break	2.3%	3.9%
Chromatid break	--	0.7%
Chromosome gap	0.3%	--
Acentric fragment	0.5%	1.2%
Chromosome deletion	0.1%	--
Chromosome exchange	0.5%	0.8%
Translocation	0.1%	--

To date, the data suggest that there is considerable variability in the frequency of structural sperm chromosomal abnormalities among normal men. The frequency of aneuploidy appears less variable. However, much more work is required on different populations to clarify the range in the frequency of numerical and structural abnormalities among normal men.

In both large-scale studies, the frequency of complements with an extra chromosome (n+1) was not significantly different from the frequency of complements missing a chromosome (n-1) although there were more hypohaploid complements in both studies. This indicates that nullisomy does occur in human gametes but that monosomic embryos are probably lost very early in development, as they are in mice (8,10) since monosomy is rarely seen even in studies of spontaneous abortions (11). Of particular interest among the aneuploid complements in our sample is a 24,Y + 1 spread. If this sperm fertilized a normal egg, a zygote with trisomy 1 would be formed. In spontaneous abortions, trisomies have been observed for all chromosomes except chromosome number 1 (4a).

Brandriff et al. reported one 24,YY complement in 909 spreads. We did not observe any 24,YY complements in 1,426 karyotypes. This is intriguing since various studies have shown that 1-5% of sperm have 2 brightly fluorescent spots, presumed to be Y bodies (13,14,22). This suggests that the fluorescent spots seen in sperm may not always reflect the presence of a Y chromosome. Further confirmation that sperm with 2 Y bodies do not correspond to 24,YY sperm is provided by the recent work of Wyrobek et al. (25). They found no agreement between the frequency of sperm with 2 Y bodies and the frequency of 24,YY sperm (based on chromosomal analysis) in semen samples from 4 men. Therefore, the fluorescent Y body technique is not a reliable indicator of Y chromosomal nondisjunction.

In our study, all chromosome groups were represented among the aneuploid sperm chromosome complements. This suggests that all chromosomes, not just the specific chromosomes which are frequently observed in trisomic abortions and newborns, are susceptible to nondisjunction. This finding has important implications for theories on the mechanism of nondisjunction. If all chromosomes participate equally in nondisjunction, then the current theories which focus only on small chromosomes or acrocentric chromosomes would be untenable. We would have to consider mechanisms which affect all chromosomes, for example, spindle attachment. However, more research is required to answer this important question since approximately 3,700 complements would need to be analyzed to detect a 2-fold increase in aneuploidy for a chromosome group containing 2 chromosomes.

The majority of structural abnormalities in both studies were chromosome breaks. Chromosome gaps, fragments, deletions, exchanges, and translocations were also observed. Brandriff et al. (5) reported that chromosome breaks at specific sites occurred more frequently than would be expected by chance alone for 3 of the 4 donors. Similar "repeat breaks" were not observed in any of the donors in our study.

X and Y chromosome ratios. The frequency of X-bearing sperm to Y-bearing sperm was 54.6% to 45.4% in our study and 51.9% to 48.1% in the study by Brandriff et al. (5). The former frequencies were significantly different from the theoretical 50:50 ratio expected, whereas, the latter were not. Future studies will determine whether individual males differ significantly in the frequency of X- and Y-bearing sperm.

Male Carriers Of Structural Chromosomal Abnormalities

Individuals who are heterozygous for a structural chromosomal rearrangement have an increased risk for spontaneous abortions and for chromosomally unbalanced children. The technique for human sperm chromosomal analysis allows us to study the meiotic segregation of chromosomes in individuals carrying a rearrangement.

We have analyzed human sperm chromosome complements in 6 men heterozygous for a translocation or inversion. Three men had a reciprocal translocation [t(11;22)(q23;q11), Ref. 18; t(6;14)(p24;q22), Ref. 2; t(5;18)(p15;q21), Ref. 2], one man had a Robertsonian translocation [t(14q;21q), Ref. 3], one had a pericentric inversion [inv(3)(p11q11), Ref. 4], and one had a paracentric inversion [inv (7)(q11q22), Ref. 16]. The frequency of chromosomally unbalanced sperm was 77% for t11;22, 32% for t6;14, 19% for t5;18, 13% for t14;21, and 0% for inv 3 and 7. The types of meiotic segregations as well as the frequency of chromosome abnormalities varied dramatically. In the reciprocal translocations t6;14 and t5;18, all unbalanced sperm were caused by adjacent 1 segregation except for one 3:1 segregation of t5;18.

Results on the 11;22 translocation have been particularly interesting. This translocation is the most common translocation observed in humans. Segregation of this translocation has appeared unusual because all liveborn unbalanced offspring (more than 80 reported) have resulted from 3:1 disjunction of the chromosomes. Various theories have been formulated as to why this translocation is predisposed to 3:1 segregation. From a single experiment, we have shown that all possible meiotic segregations (alternate, adjacent 1, adjacent 2, 3:1) do occur for this translocation. Our work demonstrates that the 3:1 segregation is not preferentially produced at meiosis, but rather that it has better survival than the other segregations, either in spermatozoa or during embryonic development.

These studies can also be used to examine whether structural rearrangements affect the segregation of other chromosomes. For example, it has been suggested that Down syndrome occurs more frequently in translocation heterozygotes, even when the translocation does not involve chromosome 21 (22,23). In one reciprocal translocation that we studied, t(5;18)(p15;q21), there was an increase in the frequency of chromosomal abnormalities unrelated to the translocation (22% compared to 8.5% in controls). Some of these abnormalities were consistent with a distributive pairing interchromosomal effect because the extra G group chromosome was similar in size to the translocated 18 chromosome.

Burns and Chaganti (7) have studied one man who was heterozygous for 2 separate reciprocal translocations, 46,XY, t(5;11)(p13;q23); t(7;14)(q11;q24). Products of alternate, adjacent 1, 3:1, and even 4:0 segregations were observed. The translocations segregated independent of each other.

Continued investigation of translocations and inversions involving different chromosomes in different men will provide us with the basic knowledge of how these rearrangements segregate during meiosis and whether the segregation of other chromosomes is affected.

Sperm Chromosome Abnormalities in Cancer Patients Treated with Radiotherapy

Radiation is known to cause chromosome damage in human somatic tissues. However, there is no information on the risk of chromosome damage in human gametes. It is important to ascertain whether radiation exposure increases the risk of chromosomal abnormalities in human gametes, since treatment of cancer patients by radiotherapy (RT) has become increasingly successful and former RT patients of child-bearing age are concerned about risks to future children.

We have studied 13 cancer patients before RT and at regular intervals after RT to determine the effect of RT on chromosomal abnormalities in sperm (20). The majority of the men (8/13) had seminoma of the testis and had undergone unilateral orchidectomy. The testicular radiation doses ranged from 0.4 to 5.0 grays.

Traditional semen analysis and sperm chromosome analysis were performed before and after RT at 1, 3, 12, 24, and 36 months. Radiation treatment had a dramatic effect on sperm count. The majority of men became azoospermic in the first year and some men never regained sperm production throughout the study. Most of the men were producing sperm 2 to 3 years after RT, but low sperm counts were common and sperm function, as measured by the ability of sperm to penetrate hamster eggs, was very poor.

Since human sperm chromosome analysis relies on sperm penetration of hamster eggs, the number of complements which could be analyzed was limited. Before RT, the frequency of sperm chromosomal abnormalities was 0%, but only 9 complements were studied. During the first 12 months, 8 complements were analyzed from 2 men who did not become sterile and one was abnormal. At 24 months, the 3 men with the lowest testicular radiation doses had sperm capable of hamster egg penetration and the average frequency of abnormalities was 12.7%. At 36 months, 86 complements were analyzed from 8 men and 18 were abnormal for an average abnormality rate of 20.9%. The frequency of sperm chromosome abnormalities was significantly higher for RT patients tested at 36 months (20.9%) ($p < .005$, two-tailed Mann-Whitney U test) than for control donors (8.5%) and for the overall frequency of sperm chromosome abnormalities post-RT (17.5%) ($p < .0002$, two-tailed Mann-Whitney U test). The range in the frequency of abnormal sperm chromosome complements in individuals at 36 months post-RT was 6% to 67%. The frequency of abnormalities detected 36 months after treatment was significantly correlated with the testicular radiation dose ($p < .02$, two-tailed Spearman's Rank Correlation Coefficient). These results demonstrate a significant, dose-dependent increase in the frequency of sperm chromosome abnormalities after RT.

Future Studies

This area of research is still in its infancy and there is a lot of important work to be done. We need to establish the range in the frequency and type of sperm chromosomal abnormalities in normal men. Hopefully, other researchers will soon become proficient at the technique and provide additional data on different populations of males.

The question of whether all chromosome groups have the same rate of nondisjunction is important in molding our theories about the mechanisms which cause nondisjunction. Since large numbers are required to answer this question, a collaborative study involving a number of laboratories may be required.

A cental question to the utility of this technique is the possible selection of sperm by the system. Studies in mice demonstrate that chromosome constitution does not influence the ability of sperm to affect fertilization (8-10). If the hamster egg acts as a barrier for some chromosomally abnormal sperm, then the frequency of chromosomal abnormalities in human sperm would be higher than our initial studies have indicated. The fact that we have found abnormalities in all chromosome groups argues against sperm selection since one would expect more abnormalities involving small chromosomes with less genomic imbalance if selection occurred. However, selection of sperm may be occurring in this system, and future studies must be designed to answer this important question.

Analysis of human sperm chromosome complements can also be used as a method to monitor the effects of environmental and occupational hazards on meiotic chromosomal abnormalities. We have demonstrated, for the first time, that radiation does cause a significant dose-dependent increase in the frequency of chromosomal abnormalities in human sperm. Many agents are suspected to be meiotic chromosomal mutagens and these will undoubtedly be evaluated in the future.

ACKNOWLEDGEMENTS

I would like to thank Kathy Burns, Judy Yamamoto, and Dianna Drews for expert technical assistance. This research was supported by grant H-75 from the Alberta Heritage Fund for Medical Research, grant MA-7961 from the Medical Research Council of Canada, project 6609-1230-53 from Health and Welfare Canada, and the Alberta Children's Hospital Foundation.

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DISCUSSION

BRANDRIFF: I would like to comment on our results and the descrepancy that we have found with Dr. Martin's data that she has just described. We have now studied 2,468 sperm from 11 normal men, and structural abnormalities are still more prevalent than numerical ones. Our results are still considerably different from Dr. Martin's. One possibility is simply chance difference between our 11 donors and Dr. Martin's 45 with regard to inter-individual differences. Another possibility that might account for these descrepancies between our two data sets is that there may be a within-individual variability, so that one might catch people at a time when the abnormalities might be higher than they are at other times. To examine this question, we resampled two of our donors at 14 months and 16 months after the first set of data that we obtained. The frequencies of both structural and numerical abnormalities are stable over time after an interval of more than a year, so that within-individual variability is probably not very great and can be discounted in accounting for these descrepancies between our two sets of data. A further possibility is that we did indeed study two different populations of donors, and since both of our laboratories studied what we called "normal" men, the question is raised: "What's a normal man?" One objective way to look at this would be to compare the lymphocytes and the sperm chromosomes. As you can see in these comparisons our donors were normal as far as the frequencies of structural abnormalities in the lymphocytes were concerned, and also as far as their sister chromatid exchanges were concerned. There were no correlations in any of these data between what we saw in the sperm and what we saw in the lymphocytes. Thus, I do not really know how to account for the difference in our data sets on normal men.

EVANS: If we put a human sperm into a hamster egg, that sperm then undergoes DNA synthesis before it shows its chromosomes. So it replicates inside a hamster background. If you could look at chromosome aberrations, you would see two kinds: chromosome-type changes, which in fact relate to events occurring earlier in the human male, and chromatid-type changes that occur in the egg itself. And the question is how many of these aberrations are chromatid aberrations made inside the egg, because then it is quite irrelevant what happens in our species. Also, what happens to the hamster chromosomes? Do you have data on the aberration frequencies in the hamster side of the matter? Does the environment of the hamster egg and human sperm predispose to chromosome abnormalities in both sets of chromosomes?

MARTIN: The chromatid-type aberrations are exceedingly rare. I've gotten one out of 1,426. Of course, if you are looking at any sort of effect of the hamster cytoplasm, it could only be on structural alterations; you would not cause numerical aberrations. We found around 3% total abnormalities in hamster chromosomes from this system--again more numerical than structural.

BRINKLEY: Has it been shown that the sperm studies by your technique are representative? Are there any data on whether those picked up are random, or does the method select for certain "viable" or "unviable" sperm?

MARTIN: My hunch is that there is no selection, but we will have to actually design experiments to look at this.

WARBURTON: We have found that by changing the method by which we collect our sperm which we use in fertilization, we are able to change the sex ratio that we see in our fertilized hamster eggs from 2:1 Y to X to 2:1 X to Y. If we keep the sperm for a second day after ejaculation, they are quite usable. We collect them by a swimming technique. It is not exactly the same as the swim-up technique that has been described in the literature. It involves collecting the ones first who swim the fastest. On Day one we find the excess of Y-bearing sperm, and on Day 2 we find the excess of X-bearing sperm. We have confirmed this now in 3 males. Small numbers, but they are significant.

HOOK: Is there a temporal variation of this type between Drs. Martin and Brandriff that might be pertinent to what Dr. Warburton just mentioned?

MARTIN: We use fresh sperm, but I know that Dr. Brandriff has been keeping sperm in some special buffer for a day or two.

BRANDRIFF: We have used sperm on Day one to compare with Day 2, and on Day one to compare with Day 3, but while we have the studies, we do not yet have the analyses.

BOND: It has been implied that centrifugation enriches for aneuploid types and that if you subject them to a swim test, then there would be less. That result, if true, is very different from the results in the mouse and Drosophila where the evidence is reasonably good that the efficiency of the sperm in fertilizing the egg is independent of the chromosome genotype. In Drosophila the sperm can function without any chromosomes at all. Is there likely to be a difference between man and the mouse with respect to the haploid genotype of the sperm and its efficiency in fertilization?

MARTIN: That is exactly why I do not think that there is likely to be any selection. I think there is a belief that the better sperm fertilize, and maybe that does happen in humans. But I do not think it is likely because of our data on the mouse and Drosophila. Certainly I thought of comparing the swim-up technique to the centrifugation technique. But logistically it becomes horrendous, because you still have to capture those sperm in the swim-up method and centrifuge them, and the numbers become very difficult to work with.

BOND: So you do not believe the implications in the literature that the swim test results in a lower aneuploid frequency?

MARTIN: The implication is there, yes, but there are no hard data and I am skeptical about it.

FORD: Dr. Martin showed a slide of one of her structural abnormalities which she called a chromosome break. I felt that it looked like a fragile site on 16q. Is this type of aberration not truly a break, but rather some type of thinning phenomenon as seen at the centromere? Have you analyzed the sites of these breaks for the possibility that they may be occurring at fragile sites?

MARTIN: Certainly, we are aware of fragile sites, and Dr. Brandriff does have some donors who have what she calls repeat breaks (i.e., breaks at the same place). After she presented that at an earlier meeting I went back and looked at our donors and found none with repeat breaks. So the breaks we see are not occurring at fragile sites or we would see more repeat breaks at the same site. But we have just recently found a man who has what appears to be a fragile site at 10q25. We see the break at this site in 3 or 4 sperm in a small sample. He has disappeared and we cannot get lymphocytes to check. But I am quite confident that he does have a fragile 10q25, particularly since it is the most common fragile site and easy to induce.

BRANDRIFF: I would like to make an update on what we call "repeat breaks" because it no longer is true that they are donor-specific; it is beginning to look as if they might be chromosome-specific. We see the same break points in the sperm from different donors. However, there has been one exception in one donor, the one that we resampled after 16 months. In the first series of experiments, we found a break in Xq1, and then 16 months later, we found a break in that same place. All the others that we used to call "repeat breaks" are more likely to be chromosome-specific. But they are not at any of the known fragile sites.

SEARLE: I want to comment on one apparent difference and one similarity between the mouse and humans. The difference is with regard to the first effects of radiation, because as far as I could gather, Dr. Martin found that one got higher frequencies of hypohaploid sperm after irradiation. Presumably these would be sperm that had been recovering the products of spermatogonia irradiation. In the mouse, from the work of Russell and, lately, Cattanach, there is no significant increase in the frequency of aneuploids at all. It is with spermatozoa that one sees an effect. The similarity is in connection with this very interesting 11/22 translocation in which there is recovery of what are essentially the trisomic types, the results of 3:1 segregation. As I understand it, this translocation is one which gives a very short and a long chromosome, and one gets exactly the same situation in the mouse (translocations giving long and short markers). Frequently, the only type which survives is the tertiary trisomics, and perhaps the tertiary monosomics, the ones with a normal complement of chromosomes with one extra little chromosome. The others all die off.

SHARMA: Is it possible to use frozen sperm in this technique?

MARTIN: We are studying this now.

WYROBEK: Was there a statistically significant elevation in the proportion of chromatid-type rearrangements 36 months after radiation? This would be an interesting phenomenon if it is true because we are dealing with stem cells that were radiated.

MARTIN: Of those cells 36 months after radiation, three of them had what we called complex rearrangements, and they are so complex you could not say how many chromosomes were there anymore.

RESNICK: Does the frequency of either aneuploidy or structural aberrations change with the age of the individual sampled?

BRANDRIFF: It just so happened that our oldest donor who was 49 years old at the time also had the highest frequencies of structural aberrations. That is an intriguing observation and we are in the process of studying men who are in their sixties. But some of our younger donors had the highest frequencies of numerical aberrations, and so that whole question of age is wide open as far as I can see.

THE ORIGIN OF ANEUPLOIDY IN HUMANS

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INTRODUCTION

Aneuploidy is the most common class of chromosome abnormality in humans and is considered by some to be the most important genetic hazard facing man (2). No less than 0.3% of all newborns are aneuploid (12), most with significant physical, intellectual, and behavioral abnormalities. Furthermore, these individuals are the least affected of all aneuploid conceptions and make up only a small proportion of this class of abnormality. The vast majority of aneuploids are spontaneously aborted, with 2/3 of all chromosomally abnormal abortions being either sex chromosome monosomies (45,X) or trisomies. Together, these two classes of abnormality constitute approximately 35% of all spontaneous abortions and, assuming a minimal rate of spontaneous abortion of 15% (22), at least 5% of all clinically recognizable human pregnancies.

Despite their high rate of occurrence and obvious clinical importance, relatively little is known about the mechanism of origin of monosomy or most trisomies. There have been several studies on the parental origin of trisomy 21 and the 47,XXY condition, but much less information is available for most other trisomies and none at all for trisomies involving autosomes without cytologically detectable variants. Similarly, there are considerable data on the parental origin of the single X chromosome in liveborns with Turner syndrome but little is known of the origin of the 99% of all 45,X conceptions that spontaneously abort.

There are two reasons for this lack of information. First, almost all studies of aneuploidy have relied on the liveborn population and therefore have been restricted to those few types of aneuploidy compatible with survival to term. Second, the ability to determine the parental origin of an additional or missing chromosome has been limited methodologically. Most previous studies have used one of two approaches, namely analysis of the X-linked Xg blood group for sex chromosome aneuploidy or analysis of chromosome heteromorphisms for autosomal trisomy. Technical factors limit the efficiency of both approaches, with successful determinations of origin being reported for only a minority of cases studied.

Fortunately, this situation is now changing, since the identification of restriction fragment length polymorphisms (RFLPs) on all human chromosomes provides a new approach to the study of the parental origin of de novo chromosome abnormalities. In this review three aspects of the study of the origin of human aneuploidy are considered. First, the necessary attributes of any system designed to study parental origin are discussed and the utility of RFLPs compared with the two other approaches that have been used; second, the information that is presently available is reviewed, including preliminary results using RFLPs; and third, questions that can now be addressed by combining cytogenetic with molecular techniques are discussed.

METHODS USED TO DETECT PARENTAL ORIGIN OF ANEUPLOIDY

In theory, the parental origin of a missing or additional chromosome can be determined by following the pattern of inheritance of any Mendelizing trait, but in reality the trait or system to be used must meet several criteria to be of practical value. The most important of these are listed in Tab. 1, with a summary of the characteristics of the three systems that have been most frequently used in studies of parental origin.

Xg Blood Group

The red blood cell antigen, Xg^a, is controlled by an X-linked dominant gene, with the two alleles, Xg^a and the nul Xg allele, having respective gene frequencies of 0.66 and 0.34 in Northern European populations (19). Phenotypically, individuals expressing the antigen are described as Xg (a+) and those without it as Xg (a-).

The highly polymorphic nature of the system makes it useful for studying the parental origin of X chromosome abnormalities, and it has been extensively used to evaluate the origin of the 45,X and 47,XXY conditions (Fig. 1). However, the approach has three serious limitations, in addi-

Tab. 1. Necessary or useful attributes of systems used to determine the parental origin of aneuploidy.

Attribute of system	Xg blood group	Chromosome heteromorphisms	Restriction fragment length polymorphisms
High level of polymorphism	Yes	Yes	Yes
Discrete variation	Yes (diallelic)	No (continuous)	Yes (di- and multiallelic)
Dosage demonstrable	No	Not applicable	Yes
Pericentromeric location	No	Yes	No
Detected in variety of tissues	No	Yes	Yes
Useful for chromosomes	X	1, 3, 4, 9, 13, 14, 15, 16, 21, 22	All

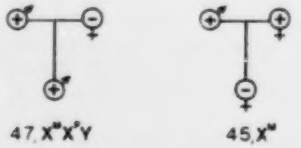

Fig. 1. Example of the use of the Xg blood group to demonstrate a paternal first meiotic division error resulting in 47,XXY conception, and loss of a paternal sex chromosome resulting in a 45,X conception (after Ref. 17). (P = paternal; M = maternal)

tion to the obvious fact that it can only be used to study X chromosome abnormalities. First, it is not possible to demonstrate dosage with Xg and, therefore, the origin of the additional X chromosome in 47,XXX individuals cannot be studied with this approach. Second, only certain parental mating types are potentially informative and the likelihood of success is rather low; for example, only 21 of 167 47,XXY individuals evaluated with Xg were informative for parental origin (Ref. 17 and P. Tippett, pers. comm.). Finally, the usefulness of Xg is limited by its tissue specificity. It is expressed in red blood cells but is not detectable in cultured cells, therefore, for all practical purposes, it is impossible to study fetuses with Xg. This has particular importance for sex chromosome monosomy, since over 99% of all 45,X conceptions spontaneously abort (13).

Chromosome Heteromorphisms

Over the past 15 years, chromosome banding techniques have become available which demonstrate the presence of variable regions, or heteromorphisms, on 10 of the 22 human autosomes. Most commonly, Q-banding, C-banding, silver staining, or a combination of these techniques is used to elucidate the heteromorphic regions. The variation consists of alterations in the size, position, or staining properties of heterochromatic regions and is ubiquitous in the human population. The variants are stably inherited and have a low mutation rate, therefore they are particularly useful for determining parental origin. Furthermore, most heteromorphisms are located at, or very near, the centromere, and are unlikely to be affected

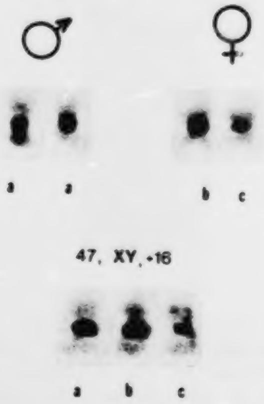

Fig. 2. C-banded chromosome heteromorphisms of parents and a trisomy 16 spontaneous abortion, showing a maternal first meiotic division error.

by crossing-over. Thus, unlike the Xg blood group or those RFLPs that are now available, chromosome heteromorphisms can be used to determine the stage of meiosis at which nondisjunction leading to trisomy occurs as well as specifying the parent of origin of the abnormality (Fig. 2).

However, there are also several limitations to chromosome heteromorphism analysis. First, the variation is not easily quantified and furthermore, the appearance of the variants depends partly on technical factors. This makes it essential that all scoring be done blindly and by independent observers but even so, it is likely that some proportion of assignments of parental origin will be in error. Second, while it is possible to distinguish between first and second meiotic division errors using chromosome heteromorphisms, it is not possible to differentiate between trisomy resulting from second meiotic division nondisjunction and trisomy due to a mitotic error early in development. Finally, the X chromosome and most autosomes do not contain heteromorphisms and, therefore, abnormalities involving these chromosomes cannot be studied with this approach.

Restriction Fragment Length Polymorphisms

Restriction fragment length polymorphisms reflect variation in the location and number of sites at which restriction endonucleases cut DNA. RFLPs have now been demonstrated on all human chromosomes, with multiple polymorphisms being reported for most chromosomes (20). The detection of allelic variation is straightforward and the DNA fragments show gene dosage, a prerequisite for determination of the parental origin of trisomies (4). The degree of polymorphism among RFLPs varies widely, but a

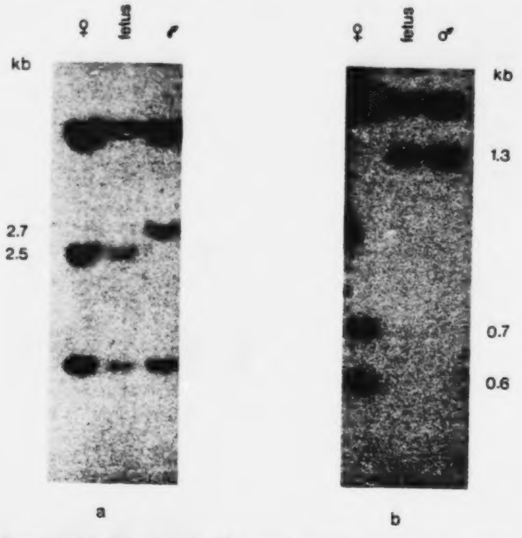

Fig. 3. 45,X abortuses in which the parental origin of the single X could be determined. (a) DNA samples were digested with Taq I and hybridized to the X-linked probe S21. The alleles of the mother are 2,2; the father, 1; and the fetus, 2; therefore, the X is maternal in origin. (b) DNA samples were digested with Taq I and hybridized to the X-linked probe 52A. The alleles of the mother are 2,2; the father, 1; and the fetus, 1; therefore, the X is paternal in origin.

high proportion is sufficiently polymorphic to be of use in studies of the origin of chromosome abnormalities (Fig. 3). Furthermore, it is clear that many more RFLPs will be identified in the next few years, and that it will be soon possible to specify the parental origin of virtually all de novo chromosome abnormalities.

The usefulness of RFLPs in studies of origin is limited in at least one respect, that is, the lack of information concerning the centromeric distances of the available RFLPs. Until chromosome-specific probes to pericentromeric regions are identified, it is possible to use RFLPs to determine the parent of origin of trisomy but not the meiotic stage at which nondisjunction occurred.

The understanding of the origin of aneuploidy will be greatly enhanced by the increasing application of molecular techniques. It will soon be possible to evaluate the origin of abnormalities for all chromosomes and, since multiple RFLPs exist for most chromosomes, to do this with a considerable degree of success. Chromosome heteromorphism analysis will continue to be useful, at least until pericentromeric RFLPs are identified. However, the practical value of Xg in studying the origin of sex chromosome aneuploidy is already less than that of a single, highly polymorphic X-linked RFLP, since Xg can only be detected among liveborns and RFLPs can be studied in any tissue or cell culture from which high molecular weight DNA can be isolated.

ORIGIN OF ANEUPLOIDY

While the use of RFLPs will undoubtedly increase our understanding of the origin of aneuploidy, information is already available from studies of chromosome heteromorphisms and Xg, and there are preliminary data from studies of RFLPs as well. In this section we summarize the available data on autosomal and sex chromosome trisomies and sex chromosome monosomy, and address three questions:

(a) Does nondisjunction occur equally among the different meiotic divisions?

(b) Does nondisjunction occur equally among the different chromosomes?

(c) Is there evidence that mechanisms other than nondisjunction result in aneuploidy in humans?

The Origin of Trisomy

Virtually all information on the origin of autosomal trisomy comes from analysis of chromosome heteromorphisms. This technique has been used extensively to study the origin of trisomy 21 among liveborn individuals, and results from over 600 families are now available (Tab. 2) (8). Maximum likelihood estimates derived from these analyses indicate that the majority of cases of trisomy 21 result from nondisjunction occurring at the first maternal meiotic division. However, errors in spermatogenesis and maternal meiosis 2 have also been identified and account for approximately 1/3 of the cases.

Limited information from studies of spontaneous abortions is available on 9 autosomal trisomies, including trisomy 21, and these are also summarized in Tab. 2. The results are consistent with those from the liveborn population in finding the most likely source of the additional chromosome to be nondisjunction at maternal meiosis 1. This holds for all trisomies

Tab. 2. Maximum likelihood estimates of different meiotic errors leading to trisomy among liveborn and spontaneously aborted trisomic conceptions (summarized from Ref. 8).

Population	Trisomy	Number of cases studied	Estimated proportion of cases due to nondisjunction at: Paternal 1	Paternal 2	Maternal 1	Maternal 2
Liveborns	21	647	0.13	0.07	0.68	0.13
Spontaneous abortions	3,4,9	21	0	0	1.00	0
	13	24	0.07	0.05	0.77	0.11
	14,15	32	0.07	0	0.93	0
	16	93	0.07	0.03	0.84	0.06
	21	37	0.25	0	0.67	0.08
	22	64	0.05	0	0.92	0.03
	mosaic trisomy	9	0	0	1.00	0

and for young, as well as older, women. Additionally, the maximum likelihood estimates for mosaic trisomies are not appreciably different from those for the nonmosaic trisomies, suggesting that in most cases the extra chromosome has a meiotic origin. This is consistent with the results of a recent study of the parental origin of mosaic trisomy 21 in liveborns (16) and with data showing a maternal-age effect in mosaic as well as nonmosaic trisomies (6), and suggests that the mosaicism typically results from a normalizing nondisjunctional event in an already trisomic conception.

Thus, it seems likely that the most common origin of any autosomal trisomy is nondisjunction at maternal meiosis 1. However, there is also evidence from the studies of spontaneous abortions for significant heterogeneity in origin among trisomies. Specifically, the estimated level of paternal nondisjunction is 25% for trisomy 21, but does not exceed 12% for any other trisomic category. Since the estimated level of paternal nondisjunction is similarly high for liveborns with trisomy 21, this implies the existence of mechanisms of paternal nondisjunction restricted to, or more likely to involve, chromosome 21. This suggestion is based on limited data on trisomies other than trisomy 21, and clearly requires confirmation. Nevertheless, preliminary data from studies of origin using RFLPs are consistent with this idea. To date, 25 trisomy 21 liveborn individuals and parents have been evaluated using RFLPs, with two of twelve informative cases being paternal in origin (1,4). Comparable data are not yet available for other trisomies, but both informative cases of trisomy 16 that have been studied with RFLPs were maternal in origin (11).

There is also indirect evidence from analyses of the incidence of trisomy and associations with maternal age indicating heterogeneity in nondisjunctional patterns among autosomal trisomies other than trisomy 21. For example, trisomy 16 is by far the most commonly occurring trisomy in humans, accounting for approximately 1/3 of all trisomic spontaneous abortions (24). By comparison, trisomies 13, 18, and 21 occur much less frequently. Nevertheless, the latter 3 trisomies are subject to much less rigorous in utero selection than trisomy 16, as evidenced by the fact that among abortuses they generally have higher gestational ages, and they occasionally survive to term. Thus, it seems likely that the high incidence of trisomy 16 derives from an increased likelihood of nondisjunction involving chromosome 16 rather than differential survival of trisomic conceptions.

Recent studies of maternal age also indicate variation in nondisjunctional patterns among different trisomies. For example, Warburton et al. (23) and Hassold and Chiu (9) observed variation in maternal age-specific rates among different categories of trisomic spontaneous abortions, with trisomies 2 and 16 showing a slow, continuous increase with maternal age, and trisomies 13 to 22 with little or no increase until age 30 yr, and a steep rise thereafter. Furthermore, trisomies 4 and 5 showed no association with increasing age in either study, and Hassold and Chiu (9) suggested that trisomy 4 may, in fact, be associated with reduced age.

Taken together, the observations on origin, age, and incidence indicate that errors in maternal meiosis 1 are the most likely source of autosomal trisomy, but that there are different chromosome-specific mechanisms of nondisjunction which may involve one or more of the four parental meiotic divisions. These two generalizations also apparently apply to the sex chromosome trisomies, information on which is summarized in Tab. 3 (3,7, 19). Direct evidence on the parental origin of 47,XXY males is available from studies of Xg in over 500 individuals and parents, and indicates that approximately 2/3 of cases are maternally derived (19). This is consistent with observations of an association between the 47,XXY condition and increasing maternal age (3). The derivation of the extra X in 47,XXX individuals cannot be studied with Xg, and results are not yet available from analyses of RFLPs. Nevertheless, the condition is associated with increasing maternal age (3) and the Xg distribution among XXX individuals is in the ultrafemale direction (19), and thus it seems likely that this abnormality is also predominantly maternal in origin.

The remaining sex chromosome trisomy, the 47,XYY abnormality, is clearly not maternal in origin since the additional Y chromosome can only be derived from nondisjunction at paternal meiosis 2. However, this abnormality does provide an apparent example of differential rates of nondisjunction involving the two sex chromosomes. That is, if nondisjunction at paternal meiosis 2 is as likely to involve the X as the Y chromosome, there should be approximately the same number of paternally derived 47,XXX as 47,XYY individuals and, indeed, the two abnormalities occur at apparently equal frequencies (Tab. 3). However, 47,XXX individuals can also arise from errors at oogenesis and the association of the abnormality with increasing maternal age suggests that this is a common occurrence. Furthermore, the frequency of maternally derived 47,XXX individuals should be approximately that of maternally derived 47,XXY individuals, assuming equal fertilization of XX oocytes by X- as Y-bearing spermatozoa. From this, it can be calculated that the frequency of maternally derived 47,XXX concep-

Tab. 3. Summary of available information on the association with maternal age, and the incidence and origin of sex chromosome trisomies (summarized from Ref. 3, 7, and 19).

Chromosome constitution	Estimated incidence among all clinically recognized human pregnancies	Origin of nondisjunction	Associated with maternal age
47,XXY	0.0008	67% maternal 33% paternal	Yes
47,XYY	0.0005	paternal meiosis 2	No
47,XXX	0.0004	Unknown	Yes

tions should be approximately 2/3 X 0.0008 = 0.0005; in other words, the same frequency as for this category as a whole. There are two possible explanations for this apparent discrepancy; first, that errors at paternal meiosis 2 are more likely to involve the Y than the X chromosome, or second, that 47,XXX individuals are more rigorously selected against than are 47,XYY individuals. As there is no evidence of differential survival between these two types of abnormality, the former explanation seems more likely to be true.

Origin of Monosomy

Information on the parental origin of human monosomy is available only for the 45,X condition, autosomal monosomies being virtually nonexistent in clinically recognized pregnancies. Data on the 45,Xs come from two sources, analysis of Xg among liveborn individuals and more recently, analysis of X-linked RFLPs among abortions (Tab. 4). Among the liveborns, data on over 300 individuals indicate the presence of a paternal X chromosome (X^P) in approximately 1/4 of cases and a maternal X chromosome (X^M) in the remainder (Ref. 19 and P. Tippett, pers. comm.). Thus, in the majority of cases the origin of the abnormality is loss of a paternal sex chromosome. Very little information is yet available on the 99% of all 45,X conceptions that spontaneously abort; nevertheless, preliminary results on a series of ten 45,X abortions indicate that, among these as well, it is at least as likely to be the paternal as the maternal sex chromosome that is missing (10).

The consistency of these observations indicates that the origin of sex chromosome monosomy is different from that of almost all other human aneuploidies in having a high frequency of paternal errors. Several explanations have been proposed to account for this, including a high level of paternal nondisjunction (21), susceptibility of the Y chromosome in Y-bearing sperm to spontaneous chromosome breakage (5), increased probability of Y chromosome loss in XY zygotes than X chromosome loss in XX zygotes (2), and random, postmeiotic loss of either sex chromosome in chromosomally normal conceptions (10). The available evidence does not discriminate between these or other hypotheses, however, the excess of X^M cases makes it clear that maternal nondisjunction is not the most important source of the abnormality. Furthermore, from considerations of the incidence of sex chromosome abnormalities it seems unlikely that either paternal or maternal nondisjunction makes a very important contribution to sex chromosome mono-

Tab. 4. The parental origin of the single X chromosome in sex chromosome monosomy. (Data from Ref. 10 and 19, and P. Tippett, pers. comm.).

Population	Technique	Number studied	Number in which origin is determined	Origin of the single X	
				Maternal	Paternal
Liveborns	Xg	306	106	77%*	23%
Spontaneous abortions	X-linked RFLPs	10	10	60%	40%

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* The values for liveborn 45,X individuals are maximum-likelihood estimates based on all 306 cases; of the 106 cases in which the origin of the single X could be unambiguously determined, 101 were X^M and 5,X^P.

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somy (5). Specifically, an estimated 1 to 2% of all clinically recognized pregnancies are 45,Xs and, assuming that nondisjunction results in equal numbers of trisomies and monosomies, it might be expected that the overall frequency of the sex chromosome trisomies would approach this value. In fact, the estimated frequency of 47,XXX, 47,XXY, and 47,XYY conceptions is only 0.0013 (Tab. 4), less than 10% of that of the 45,Xs. This cannot be explained by in utero selection against the sex chromosome trisomies, as available evidence suggests that the vast majority of such conceptions survive to term. Thus, it seems reasonable to assume that all but a fraction of 45,X conceptions arise from a process of sex chromosome loss that is unrelated to meiotic nondisjunction.

There is some evidence to suggest that this process may occur postmeiotically. For example, in the mouse, studies of irradiated pronuclear eggs suggest that the male pronucleus is particularly susceptible to loss of the X or Y chromosome (18). Furthermore, there is evidence of a postmeiotic etiology among liveborns with Turner syndrome, since at least 25% of all individuals with a 45,X cell line carry a chromosomally normal line (13). In fact, Hook and Warburton (13) have suggested that all liveborn 45,Xs may be mosaics for a normal cell line in some organ or tissue, with the high in utero lethality of the 45,X condition being due to the absence of a normal line in almost all such conceptions.

SUMMARY AND FUTURE APPLICATIONS OF STUDIES OF PARENTAL ORIGIN

The available data make it possible to draw several conclusions about the origin of human aneuploidy. First, it is clear that the most likely source of the additional chromosome in human trisomy is nondisjunction at the first maternal meiotic division. This is probably the case for all trisomies, excluding the 47,XYY condition, for mosaic as well as nonmosaic trisomies, and at all maternal ages. Second, the combined evidence from studies of origin, incidence, and maternal age indicates the presence of different chromosome-specific mechanisms of nondisjunction. Thus, trisomy 21 is apparently associated with an increased level of paternal nondisjunction and, among the remaining trisomies, there is evidence for at least two different, maternal age-related mechanisms of nondisjunction (23). Finally, the results of studies on sex chromosome monosomy demonstrate a mechanism of chromosome loss whose genesis is unknown but that appears to be unrelated to meiotic nondisjunction.

In addition to providing important information on the origin of human aneuploidy, the studies summarized in this chapter have raised basic questions concerning the origin of aneuploidy that can now be addressed by combining molecular with cytogenetic techniques. For example, it has been suggested that the presence of a normal cell line differentiates spontaneously aborted from liveborn 45,X conceptions. This hypothesis can now be rigorously tested, since the application of RFLPs makes it possible to directly compare the levels of mosaicism between these two categories of monosomy. Additionally, observations on maternal age indicate the existence of chromosome-specific patterns of nondisjunction, but as there are few data on the origin of most human trisomies it is not known if this variation is associated with one, or different, stages of meiosis. The identification of RFLPs on all human chromosomes makes it possible to compare the mechanism(s) of origin of all trisomies and thus to determine the nature and extent of variation in nondisjunction involving different chromosomes.

It is also anticipated that future studies of parental origin will provide important information on the etiology of human aneuploidy. In this regard, 3 recent reports on the parental origin of trisomy 21 are of particular interest. In the first, Mikkelsen et al. (15) described a possible association between place of residence and nondisjunction. Specifically, paternal errors and maternal second meiotic division errors were observed more frequently in urban than rural settings, suggesting a possible association between environmental agents and nondisjunction occurring at these stages. Recently, Jackson-Cook et al. (14) described another factor that may predispose to nondisjunction involving acrocentric chromosomes, namely the presence of double nucleolar organizing regions (NORs) on one or more of the acrocentrics. They identified a double NOR in 14 of 50 parents of Down syndrome individuals but observed none in a control population of 45 individuals. Furthermore, in each relevant case in which the parent of origin could be determined, the nondisjunctional event occurred in the parent carrying the double NOR. Finally, Antonarakis (1) recently reported a possible genetic predisposition to nondisjunction of chromosome 21. Thus, he identified a specific chromosome 21 RFLP haplotype which occurred more frequently among parents of Down syndrome individuals than in a control population.

The results of each of these studies have far reaching implications and the latter two reports in particular have attracted widespread attention in the media. It is important to note that the observations are as yet unconfirmed, and that independent confirmation is essential before they are applied in any genetic counselling situation. Nevertheless, the approach used by these studies may eventually result in the identification of etiologic factors associated with specific meiotic errors. Continued use of RFLPs will greatly enhance the likelihood of identifying and characterizing such effects, since all aneuploid conditions will be amenable to study.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant HD-07879. Additionally, the work was done during T.H.'s tenure as a National Down Syndrome Society scholar.

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DISCUSSION

HASSOLD: I want to voice my concern about chromosome heteromorphism analysis, particularly with respect to the idea of relaxed selection. In my experience with chromosome heteromorphism analysis, I find it an extraordinarily difficult technique and one in which I think it is very easy to make misassignments of parental origin. I think that the application of RFLPs, a very objective test, rather than the subjective chromosome heteromorphism analysis, should over the next few years, sort out the relaxed selection idea.

BOND: Is there a maternal-age effect for the subclass of the 45,X cases which are known to arise from a maternal error?

HASSOLD: The numbers are very small, but at one time I looked at the data I had and there was no obvious difference.

FORD: At a recent meeting on the chorionic villi biopsy, many people reported that when they found trisomy 16 in the chorionic villi, it was with a normal embryonic karyotype. Would you speak about the possible bias in trisomy 16 studies?

HASSOLD: Almost all of the samples that we get with trisomy 16 are placentally derived; that is the only tissue that we see. So we really do not have any data on that point.

WARBURTON: In all the spontaneous abortions that we have looked at that have trisomy 16 (and we have looked at many where we get the whole specimen that is present; it is not always the whole conceptus, but it is all that is available), I have never seen an embryo which looked anything like it was normal, and which would have passed the ultrasound test for fetal viability. My guess is that, perhaps, in some of the instances of the discrepancy between chorionic villi and embryo, the embryo and the villi biopsies did not really go together. Perhaps there were twins and the trisomy 16 embryo was lost. Essentially, it was a normal twin embryo studied cytogenetically. I just cannot believe that one could have a placenta with trisomy 16 and the corresponding embryo which looked normal, having seen literally hundreds of instances in which I have never seen a normal embryo associated with the trisomy 16 placenta.

HOOK: But one must then always have sexual concordance to these postulated twins and, thus, they are presumably monozygotic. Otherwise, sex discordance would have been detected previously.

ASHLEY: In mice as in man there is a great excess of XO over XXY or XXX. There is a higher frequency of paternal X loss than maternal X loss. In synaptonemal complex studies I have found cases where the entire sex body appears to have been lost from a pachytene nucleus. I do not have statistics on this, but if the whole sex vesicle is lost, so that you have lost both the X and Y, you now have a nondisjunctional means of arriving at an O sperm, and therefore, a XO zygote that would have a paternal origin.

HULTEN: We have not seen any loss of the XY complex in more than 1,000 spermatocytes from the human.

ASHLEY: Our observation is that if we find a mouse that has one sex body lost, we will find it in several others within that individual. So it may be that it has a genetic component, or it could possibly be a preparation artifact.

HULTEN: I have found in our clinical material some cases of 45,X in which there is a structural rearrangement of the Y in the father. Have other people seen this? If so, one could then assume that structural rearrangement would make this chromosome more prone to become lost, either zygotically or postzygotically.

BERNSTEIN: We have been doing a study on a polymorphic inversion of a Y which we have found in a particular Muslim Indian group which seems to be a founder effect, and we have not ever observed any sex chromosome abnormalities in their offspring. We have already done over a hundred cases.

MIGEON: Dr. Hassold, would you comment on the effect of sex ratio and trisomies?

HASSOLD: In our study, trisomy 21 does have a significantly increased sex ratio by comparison with the other trisomies, and among liveborns, there is pretty good evidence for an increased level of XYs among trisomies by comparison with the general population. We did a very quick analysis of sex ratio among trisomy liveborns in which the stage of meiosis and apparent origin was known. It did, in fact, look as if there might be an increased sex ratio largely restricted to the paternal 1 cases. Is this due to a 21-Y pairing of some type? That is a very speculative statement that obviously requires confirmation.

GENEROSO: In humans the oocytes are arrested somewhere in meiosis 1, and therefore, the older the woman gets, the longer the oocyte sits in that state. In trisomics, like trisomy 21, is there any indication that the maternal effect is on either meiosis 1 or meiosis 2, or are both affected to the same extent?

HASSOLD: As I recall there is very little difference in the effect of maternal age on any of the stages. I think that it is something that will have to be sorted out with more data.

MASON: Early evidence in Drosophila suggested that there was a significant frequency of second division nondisjunction. But this has been shown to be the result of first division nondisjunction associated with recombination between the marker and the centromere. Dr. Hassold, how close are your markers to the centromere, and can you distinguish between first and second division nondisjunction?

HASSOLD: There is not a great deal of evidence on that point. I think probably the evidence that is cited by most people in this field is Dr. Hulten's work on chiasmata in the male, which indicates that there is unlikely to be a great deal of crossing over between the centromere and markers that we are using. But there are very few hard data to support the assumption that we all make in this kind of a study, which is that there is minimal crossing-over.

MATERNAL AGE, PATERNAL AGE, AND HUMAN CHROMOSOME ABNORMALITY: NATURE, MAGNITUDE, ETIOLOGY, AND MECHANISMS OF EFFECTS

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INTRODUCTION

An association of advanced maternal age with the trisomy 21 phenotype, Down syndrome, has been known for many years and has been found in all ethnic, racial, and socioeconomic groups investigated. (For references see Ref. 25 and 41.) More recently, an association of advanced maternal age with other chromosome abnormalities, both viable and nonviable, has been reported. Maternal-age effects and their mechanisms are discussed extensively in sections that follow.

Any association with advanced maternal age theoretically may be indirectly attributable entirely to other factors, e.g., paternal age or high birth order. The possibility that paternal-age effects account for the entire observed parental-age association with Down syndrome was excluded by Penrose and Jenkins in 1933 (31,39) and only recently for 47,+13, 47,+18, and 47,XXY by others (6,9,28). For the other trisomies we have little evidence because of sparse data. Nevertheless, the possibility that paternal age makes a small contribution independent of maternal age for 47,+21 has recently been an issue of some controversy. This is discussed in the paternal-age section of this chapter.

High birth order has been excluded as accounting for the maternal-age association for 47,+21 (41) and the X trisomies (6). Of interest, there is some suggestive evidence that primogeniture (1st birth order) may have a slight association with Down syndrome, an effect contrary to any parental-age effect. But it is possible this primogeniture effect is due to ascertainment bias in some studies. (See Ref. 23 and 25 for references and further discussion of these views.) Birth order has not been extensively investigated for most abnormalities and is not considered further here.

MATERNAL AGE AND 47,+21

The association of advanced maternal age with human trisomy, especially 47,+21, is well-known, and has been reported upon and reviewed extensively elsewhere (e.g., 23,25,31,39,41). Ninety-five percent of Down

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syndrome is attributable to 47,+21; the remainder is attributable to structural abnormalities with little association with age. For this and other reasons (28) the changes with age of the Down syndrome phenotype reflect almost exclusively the 47,+21 contribution. But 47,+21 is not the only abnormality whose effect varies with age. (See next section for discussion of other abnormalities.)

In statistical evaluation of the advanced maternal-age effect one must consider the theoretical possibility that the distribution of maternal ages of those with Down syndrome is "shifted to the right" of the distribution for those of all livebirths. Another possibility is that only some proportion of Down syndrome cases have this tendency to association with advanced maternal age and some do not. An examination of the relative frequency of Down syndrome at various ages by Penrose revealed evidence for 2 modes in the distribution: one at the mode for all livebirths, the other at a higher age. This led Penrose to postulate 2 numerically significant, different categories of Down syndrome: one maternal age-dependent, the other maternal age-independent (41). (The relative contributions of these 2 postulated components are discussed further below.)

Nevertheless, despite the heuristic attractiveness of this explanation for the bimodal (or biphasic or bitangential) distribution of maternal ages for Down syndrome, Moran (37) has noted that this phenomenon may well be, in effect, a statistical "coincidence." It may result simply from a marked increase in rates of Down syndrome in the later childbearing years, the same ages in which a very sharp drop in fertility occurs. The relative distribution of Down syndrome is a function of both the maternal age-specific rates of an affected child and of age-specific fertility. If as age increases the former rises more steeply than the latter drops, then a secondary peak (or shoulder) at the older ages may result.

The model above is based only on the relative distribution of Down syndrome and normal livebirth at different ages. It tells us nothing about the absolute rates of Down syndrome and how they vary with maternal age.

Graphs (Fig. 1) of maternal age-specific rates (Tab. 1) indicate that qualitatively, 3 components in the change of rate with age of 47,+21 may be described (23,25). From age 20 to about 30 yr, the rate increases only slightly. Above age 30 yr the rate of change increases exponentially and achieves markedly high rates above 40 yr. Below age 20 yr, there is some fluctuation and heterogeneity among studies. In some, the nadir of the rates appears to be at age 19 to 20 yr, and the rates are higher under this age (11,23,25,56). At age 15 or 16 yr the rate may be as high as at age 30 yr in some jurisdictions. The observations on teenagers are not ubiquitous however (see data in Ref. 23 and 25), and they may result not from biological differences, but perhaps from reporting errors or from social factors (such as increased childbearing by retarded young mothers, some of whom are 47,+21 mosaics). As the change, if any, is relatively modest and not clearly of true biological origin, I will not discuss further this possible young mother-age effect.

A question of much greater significance, both biologially and socially, is why the observed rate rises with age so markedly after age 30 yr. It has been suggested that the observed data for Down syndrome over age 19 yr may be explained by a "constant plus exponential" model (CPE), i.e., superimposition of 2 different contributions, one constant, the other increasing (first order) exponentially after age 20 yr (33,34). This results in an equation of the form $\underline{y} = \underline{a} + \exp(\underline{bx} + \underline{c})$ where $\underline{y}$ is the rate, $\underline{x}$ is maternal age, and $\underline{a}$, $\underline{b}$, and $\underline{c}$ are constants. (In most studies $\underline{b}$ is about 0.25 to 0.30, $\underline{a}$ about 5.7 to 7.1×10^{-4}, and $\underline{c}$ about -16.1 to -17.7.) Parameters derived from the observations on several different data sets

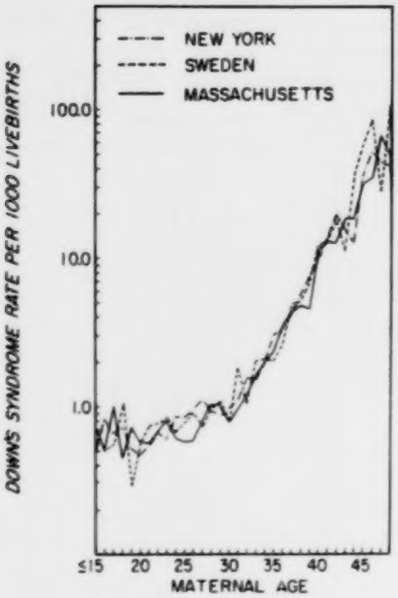

Fig. 1. Graphs of rates of Down syndrome per 1,000 livebirths by 1-yr maternal-age interval in 3 independent studies. For sources see Lamson and Hook (33,34) or Hook (23,25).

result in equations that, when graphed, agree with the observations of a slow increase up to age 30 yr and an apparent rapid change thereafter. In fact, age 30 yr is just where the two different postulated components intersect, i.e., the age at which the postulated maternal age-dependent and age-independent components are roughly equal. Thus, despite the apparent observed change at age 30 when the rates are graphed, there is no necessary alteration in biological processes at that age, although certainly there may be (see discussion in Ref. 23).

The CPE model provides an attractive mathematical formulation for the postulation by Penrose of significant maternal age-independent and maternal age-dependent components of Down syndrome--the $\underline{a}$ and $\underline{b}$ parameters, respectively, in the equation above. But I emphasize that other types of equations may also account for the observations and the apparent alteration at age 30 yr in maternal age-specific rates, so there is no proof that the CPE model holds. (See Ref. 34 for discussion of other models.)

In most contemporary populations of developed countries today, the proportion of the putative age-dependent and age-independent contributions may be calculated as about 35% to 50% maternal age-dependent, and 50% to 65% maternal age-independent. (Of course, these proportions vary with the maternal-age distribution of all livebirths. Moreover, these estimates ignore any effect of prenatal diagnosis and selective abortion of trisomic fetuses in older mothers.) Thus, both putative components are important etiologically.

The inferred age-independent component includes rare cases of translocation Down syndrome and of 47,+21 cases born to parents who are carriers of 47,+21 lines. But because of the numerical estimates above, there must

Tab. 1. Rates of chromosome abnormalities per 1,000 livebirths at varying maternal ages (in absence of amniocentesis and selection abortion).

Maternal age (yr)	Down syndrome*	All clinically significant abnormalities**
15	1.0	2.2
16	0.9	2.1
17	0.8	2.0
18	0.7	1.9
19	0.6	1.8
20	0.6	1.9
21	0.6	1.9
22	0.7	2.0
23	0.7	2.0
24	0.8	2.1
25	0.8	2.1
26	0.9	2.1
27	0.9	2.2
28	1.0	2.3
29	1.0	2.4
30	1.1	2.6
31	1.1	2.6
32	1.3	3.1
33	1.7	3.5
34	2.2	4.1
35	2.7	5.6
36	3.5	6.7
37	4.5	8.1
38	5.7	9.5
39	7.2	12.4
40	9.2	15.8
41	11.7	20.5
42	14.9	25.5
43	19.0	32.6
44	24.2	41.8
45	30.8	53.7
—	—	—
—	—	—
—	—	—

* Rates are about 10% above lowest limit of ranges cited in Ref. 24.

** Excludes 47,XXX which also has a strong maternal-age association, but one not precisely defined at present (see text). Rates given are from Hook (24).

also be many 47,+21 individuals born to ostensibly normal parents who are in the inferred maternal age-independent class. Are such parents cryptic gonosomal mosaics for 47,+21 lines? We do not know, but it is of interest that the recurrence risk for 47,+21 to a young mother is 10-fold greater than the population rate, suggesting such cryptic mosaicism may be present in at least some young parents. It is also suggestive that a trend to advanced grandmaternal age has been reported (40), consistent with the hypothesis that the original age-dependent event occasionally is in the maternal grandmother, and an (ostensibly) normal mother may be a cryptic gonosomal mosaic for 47,+21, having lost the extra chromosome in most of the other tissues (see also Ref. 1 and 38).

THE ORIGIN OF THE MATERNAL-AGE EFFECT

The explanation for the age-dependence of rates is unknown. Theoretically there could be a prezygotic origin and/or a zygotic and postzygotic origin of the association with maternal age. Prezygotic processes could include such factors as possible age effects upon nondisjunction of chromosome 21 or upon relative survival of +21 gametes. Zygotic and postzygotic effects could include maternal-age influences upon ability of +21 gametes to participate successfully in fertilization, or upon survival of +21 zygotes, embryos, or fetuses (i.e., postzygotic "relaxed selection").

Recently studies of parental origin of the extra +21 chromosome revealed a paternal origin of the extra +21 in about 20-25% of the instances of 47,+21. But contrary to expectation, there was not a markedly lower parental age in these cases (either maternal or paternal) than in those of maternal origin. If there is no maternal-age effect for 47,+21 of paternal origin, then randomly selected instances in most populations of developed countries today should have mean maternal ages of 25 to 26 yr. Cases of maternal origin should have mean maternal ages of 30 to 32 yr (if selected randomly), so the expected difference should be +4 to +7 yr. But this is not found in most studies (Tab. 2). (See Ref. 11, 27, 36, and 49.)

This observation has several theoretical explanations: (a) a paternal- age effect upon cases of paternal origin which results indirectly in a maternal-age effect; (b) technical errors in assignment of origin in some studies; (c) maternal-age effects upon the fertilization of +21 gametes; (d) "relaxed" postzygotic selection effects of some type; (e) unrecognized ascertainment bias; or (f) chance. There are no grounds for a paternal-age effect for Down syndrome of any magnitude sufficient to account for this observation (see last section of this chapter). With regard to technical errors, probably some have been made in assignments, but it remains only speculative that such can account for the observations in Tab. 2.

Tab. 2. Comparison of maternal ages for cases of 47,+21 in which the extra chromosome is of maternal or paternal origin. Results of 8 studies (see references in Hook [27]).

	Maternal origin			Paternal origin			
Senior author*	n	mean	S.D.	n	mean	S.D.	Difference
del Mazo	22	33.8	7.3	7	36.4	8.0	-2.6
Mattei	41	29.6	6.3	10	30.2	6.0	-0.6
Uchida**	105	32.2	6.7	34	31.1	8.6	+1.1
Mikkelsen	67	31.3	7.6	13	29.3	5.8	+2.0
Magenis	33	30.2	7.7	9	27.9	7.7	+2.3
Hatcher	34	30.9	6.7	9	26.2	6.1	+4.7
Manning	8	31.0	6.3	4	25.3	5.1	+5.7
Wagenbichler	10	37.1	7.0	8	28.8	7.9	+8.3
Expected***		30-32			25-26		∼+5.5

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* See References in Hook (27).

** Personal communication, 1985.

*** Expected on the assumption of no maternal-age effect for cases of paternal origin and that the cases studied are representative of the affected population. The expected values vary with the maternal-age structure of childbearing populations. Values given are for the recent studies in "developed" regions.

With regard to selection, earlier this had been raised as a theoretical possibility or hypothesized because of the apparent lack of age differences between cases of maternal and paternal origin. But recently Ayme and Lippman-Hand (3-5) claimed that they had direct evidence for such an effect in spontaneous abortus data on 47,+21 alone, and also for all trisomies. Unfortunately, their analyses pooled data on viable and nonviable trisomies and thus was highly questionable, both with regard to inferences for any specific trisomy or for all trisomies together (8,55). In addition, other data, such as those indicating a higher mean maternal age among +21 livebirths and a lack of a maternal-age effect for D/21 Robertsonian translocations associated with liveborn Down syndrome, are also contrary to their claim (27).

This negative evidence does not disprove the possibility that selection is responsible for any maternal-age effect, but indicates only that if selection occurs, then it occurs prior to the usual first recognition of pregnancy and identification of spontaneous abortion, i.e., before about 4 to 5 menstrual (or 2 to 3 conceptional) weeks of gestation. In older mothers it is still possible there is preferential survival or fertilization of +21 gametes, or else there is preferential in utero survival of +21 conceptuses prior to the gestational ages at which spontaneous abortuses with +21 have been collected in studies to date. Indeed, these remain the only apparent explanations for the maternal ages in paternal origin data cited above, aside from technical errors, chance effects, or unrecognized ascertainment bias.

There have been other hypotheses for the aging effect in humans, including delayed fertilization in older women because of diminished frequency of coitus (17), persistence of nucleolar organizers in older women (14), and diminished chiasma frequencies and decreased univalents with maternal age (19). All of these assumed that the age effect is mediated by effects upon nondisjunction, although German's theory (17) could be modified to allow for postmeiotic selective effects upon fertilizability. For none of these is there definitive evidence that they account for the maternal-age association in humans. For reports counter to German's suggestion, see, e.g., Ref. 35 and 41a.

For evidence against the significance of the nucleolus, note that there are aging effects for trisomies of non-nucleolar chromosomes, e.g., trisomy 18 (see next section). In particular, the graph of change in rate of +18 with age is almost exactly parallel to that of +21 (22). I know of no data bearing on chiasma frequencies in human females.

MATERNAL-AGE EFFECTS FOR OTHER HUMAN ANEUPLOIDY

There is much less known about age effects of other types of aneuploidy. These have been studied in much less detail than 47,+21 and Down syndrome and less information is available.

Under age 20 yr there are a few data. From age 20 yr on, some trisomies appear to have maternal-age effects similar, though not identical, to 47,+21, although a contribution of a paternal-age effect has not been excluded in most of the studies discussed below. Only for a few has the possibility that paternal age accounts for all of the association with maternal age been excluded. This has been done for 48,+18, 47,+13, 47,XXY, and 47,XXX (6,9,15,22,26,34a,34b,45). 47,+18 appears to be parallel to 47,+21 up to about age 45 yr at least (22), but then may tend to level off (15,26). 47,+13 increases with age, but not as markedly as 47,+18 or 47,+21, and levels off at around age 42 or 43 yr (15,26). The abnormalities involving extra X chromosomes increase with age, but apparently not as markedly as 47,+18 or 47,+21 (45), although another model implies marked increases at the extremes of age (6). Under age 30 yr the latter analysis suggests that the rates of 47,XXX may be higher than at age 30 yr.

In spontaneous abortuses one analysis has revealed that most of autosomal trisomies have patterns grossly similar to that of 47,+21 in livebirths, with a relatively modest change of rate with age, if any, up to age 30 yr and a marked increase after that (55). Thus, these probably may all be modeled by a CPE equation of the type described above, $y = a + \exp(bx + c)$. Reported exceptions are trisomies 1, 2, 3, 4, 5, and 16.* Trisomies 2 and 16 in spontaneous abortuses are reported as showing a slow, continuous rise with age from 20 yr on, with no apparent change at 30 yr. Trisomies 3, 4, and 5 in spontaneous abortions exhibit no apparent maternal age-dependent association. And there are no data whatsoever on trisomy 1 from abortuses. (Despite the above, it is conceivable that for trisomy 2 and 16 there might be a putatively constant, but very small, age-independent effect dwarfed by a maternal age-dependent effect for these conditions. Conversely, for trisomies 3, 4, and 5, a small age-dependent effect might be dwarfed by a large age-independent effect [55a]. That is, despite the apparent subjective impressions, it is possible that CPE equations of the type $y = a + \exp[bx + c]$ might be found to fit the observations on trisomies 2, 3, 4, 5, and 16, with appropriate choice of parameters a, b, and c. It will be of interest to see what further analysis reveals.) Data on mean ages in trisomies in abortuses appear in Hassold and Jacobs (17f) and Hassold et al. (18).

47,XYY, in which the extra chromosome necessarily results from 2nd division *paternal* nondisjunction (or mitotic nondisjunction), appears to have little association with parental age and may even have a negative (downward) trend at older ages (6).

For monosomy-(X) there appears to be a small negative association with maternal age, both in livebirths (6) and spontaneous abortuses (54). (This appears unlikely to result from a paternal-age effect [see below].) Monosomy may result from anaphase loss as well as from nondisjunction, and moreover, may be more likely than trisomy to result originally from mitotic events. (The reasons for this are that there are many 45,X/46,XY and 45,X/46,XX mosaics reported which almost certainly resulted from some errors at mitosis. And it is likely that some apparently nonmosaic 45,X conceptuses are either cryptic mosaics of this type or started as such mosaics and then lost the normal 46 line.) Most commentators have assumed that because the prevalence of 45,X is so much greater than the other products of sex chromosome nondisjunction (47,XXY and 47,XXX), that anaphase loss or similar events leading to sex-chromosome loss must account for a much larger proportion of 45,X than does nondisjunction. This difference in etiology might account for the difference in maternal-age effects. That is, nondisjunction resulting in 47,XXY and 47,XXX and a very small proportion of 45,X could perhaps be influenced by advanced maternal age, but anaphase loss and other factors resulting exclusively in 45,X might be relatively independent of maternal age. (It is also of interest that the X chromosome usually missing in 45,X is the paternal chromosome, implying that if meiotic nondisjunction contributes to this condition it is predominantly paternal nondisjunction.) Nevertheless, as discussed above with regard to 47,+21, it is also possible that differential selective effects of maternal age upon conceptus survival account for the differences in maternal-age effects between 45,X and the two trisomies, 47,XXX and 47,XXY.

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* Trisomies 6, 8, and 12 also appear to be exceptions in view of the mean maternal ages reported by Hassold et al. (18).

With regard to structural chromosome abnormalities there are relatively few data. There are a large number of different categories of events (deletions, rings, reciprocal translocations, Robertsonian translocations, supernumerary markers and fragments, and isochromosomes, among others), each of which could have a different maternal-age effect depending on chromosome and breakpoint(s) involved. For supernumerary markers, e.g., 47,+mar, there is strong suggestive evidence for a maternal-age effect at amniocentesis, with a mean age of 38.4 ± 3.6 yr in 19 cases compared to a control figure of 35.6 ± 4.3 yr in 26,558 controls (27a). For other categories, there are no significant trends, but the numbers involved are quite small (see Ref. 27a). Van Dyke et al. (52) cite very suggestive evidence in reported cases in the literature--mostly from livebirths--of a maternal-age effect for inverted duplications, with mean maternal ages of 29.2 to 35.7 yr in various categories compared to control ages of 26 to 27 yr.

EXPERIMENTAL EFFECTS IN ANIMALS ON MATERNAL AGE

In view of the uncertainty as to whether the maternal-age effect for trisomy in humans is mediated by effects on nondisjunction or on selection, work on this issue in experimental animals is thus of great interest. Nevertheless, there is considerable question whether such work provides a model for aneuploidy in humans. For example, there was no evidence of aneuploidy at "term" (i.e., the time of livebirth) in mice in one study (17c) contrary to the observations on human livebirths. Earlier in mouse pregnancy, age effects have been reported (17d,55b; see also Ref. 14a and 49a). There has been a great deal of hypotheses and work which has assumed that this age effect earlier in gestation (and the observed age effect in humans) was mediated by age effects on nondisjunction. Yet there is inconsistent evidence on this in mice. Uchida found suggestive evidence for an age effect on nondisjunction (49b,50a), e.g., in one study there were 0/1,054 hyperploid oocytes at metaphase 2 in younger mice and 4/1,302 in older mice. But Golbus (17a,17b) found no difference between young and old mice, as did Polani and Jagiello (41b) and Martin et al. (34d). To complicate matters, Martin did observe higher levels of evidence for nondisjunction in "middle-aged" mice (6/110 vs 0/187 and 0/155 in young and older mice). For whatever light these variable and inconsistent data shed on the human situation, there appears to be at least some evidence for selective effects in mice accounting for a putative age effect, a view explicitly endorsed by at least one worker (17a,17b).

It is possible that the differences among these studies are attributable simply to errors in some of them, or alternatively, to a heterogeneous and variable effect in mice associated with many idiosyncratic variables not yet recognized. The latter interpretation, however, makes it very difficult to use any of such mouse work on aging as a guide to what occurs in humans. We don't know which animal experiments are appropriate and which are inappropriate to the human situation; it is only further study of humans that will clarify the significance of the animal work.

It is worth emphasizing again that a strong maternal-age effect for +21 in livebirths in humans has been observed in all racial and socioeconomic groups reported on to date.

PATERNAL AGE

This is a controversial factor because of variable and inconsistent results, particularly with regard to Down syndrome and 47,+21. As noted above, earlier investigations of the association of parental age with Down syndrome (31,39) concluded that maternal age was much stronger than any putative paternal-age effect. Subsequently in many of his writings, Penrose, we well as others, appeared to assume that paternal age was not of significance (see, e.g., Ref. 41), although at least some observers pointed out that a modest paternal-age effect could not be excluded from consideration after analysis of the available data (34c). The issue was reopened recently with the discovery that in about 20% of Down syndrome cases the extra chromosome can be shown to be of paternal origin (see, e.g., Ref. 17e). Despite the fact that there is no positive parental-age effect for the XYY genotype (see, e.g., Ref. 6) in which the extra chromosome is known to be of paternal origin, this discovery led some to the apparent inference that paternal age independent of maternal age must be a significant risk factor for Down syndrome (19b).

One of the limitations of such investigations is that maternal age and paternal age are so highly correlated that it is very difficult to demonstrate a modest effect of one variable in the face of a strong effect of the other. In addition, maternal age-specific rates of Down syndrome rise rapidly with maternal age while fertility plunges quickly (see, e.g., Ref. 23), leading to possible statistical artifacts. As an example, one of the first recent studies of paternal age since the discovery of patroclinous, extra 21st chromosomes was that of Stene et al. (46a) who claimed "statistically significant" evidence for a 2-fold paternal-age effect for cases born to men 55 yr and over. While no evidence was reported for an effect in men younger than this age, they also claimed a strong paternal-age effect at ages below 55 yr. Their analysis, however, was by <35, 35-39, and ≥40 yr maternal-age intervals. Erickson (11) showed that he could construct an artifactual paternal-age effect using the methodology of Stene et al. (46a) applied to another data set. This disappeared when he used a more appropriate method carrying out an analysis by 1-yr maternal-age intervals. Similarly Matsunaga et al. (35a) reported statistically significant evidence for a paternal-age effect for men 55 yr and over in data analyzed by 5-yr intervals, but Lamson et al. (33a) showed that the methodology used here could produce artifacts which disappeared when analysis was by 1-yr intervals.

Investigations in livebirths by other investigators in general differ from those of Stene et al. in not revealing as strong a positive effect. Nevertheless, they also differ among themselves. Some have reported a significant negative association (42b), some found no evidence for a paternal-age effect (11,24a,42a), some found a suggestive but not significant effect (12), and others found significant effects, albeit not of strong magnitude (24a,13). One study suggested that a weak effect of the order of about 1% increase in risk with each year of paternal age was consistent with the observations (24a), and tables have been constructed projecting what these expected rates should be at various combinations of maternal and paternal ages (26). Even this may be too strong an effect in view of the later results of Roecker and Huether (42b). But no studies in livebirths to date done by 1-yr intervals have revealed effects as large as those claimed by Stene et al. (46a). Other evidence comes from a search for a paternal-age effect in 47,+21 cases in which the extra chromosome is of paternal origin. There was no evidence for such in the livebirth instances reported in the literature (29).

Stene et al. (46b) also published data on 60 cases of 47,+21 diagnosed prenatally at amniocentesis and claimed a very strong paternal-age effect similar in magnitude to that claimed by Stene et al. (46a), which analyzed livebirth data by 5-yr intervals. They have projected from these observations putative risks for women of any age married to men of 41 yr or older sufficient to justify amniocentesis (46b). Nevertheless, other larger-scale reports on paternal age at prenatal diagnosis and 47,+21 found no evidence for a significant paternal-age effect independent of maternal age.

These include a study in New York State on 101 fetuses with 47,+21 (26), a collaborative West European study on 161 cases (15), and a report from France on 118 cases (43).

The strong positive results of Stene et al. have received a good deal of attention and have been widely cited in textbooks (e.g., Ref. 47) to justify their idiosyncratic position. Nevertheless, there is such consistent failure to repeat the strong trends of their studies that I believe they must be regarded as simply peculiar "outliers" which do not reflect ubiquitous biological factors.

It is possible that there is some type of unusual temporal and/or geographic variation in paternal-age effects, but the nature and extent of these are still to be defined if they exist. (Perhaps they are present particularly in the area studied by Stene et al.)

At the present time, no inferences are warranted about any paternal-age effect for Down syndrome or 47,+21. And as noted above, data on other trisomies in livebirths (47,+18, 47,+13, 47,XXY, and 47,XXX) are also negative (6,9,28). (A suggestive effect for 47,XXY at amniocentesis has been observed, however, by Ferguson-Smith and Yates [15]).

In spontaneous abortuses there has been relatively little analysis. In an as yet unpublished talk, Hatch (18b) noted the results of 2 phases of a New York study: one from April 1974 to July 1979, the other from August 1979 to November 1982, each with about the same number of cases. In phase I (also summarized in Ref. 18a) strong paternal-age effects were observed after adjustment for maternal age for all trisomies together (+1.5 yr at the younger maternal ages, +3 yr at the older ages, with little effect in between) and for monosomy (45,X), about +2 yr. The latter effect is despite the apparent lower maternal age associated with this condition discussed above. In phase II, there was no effect or a negative effect for all trisomy and only a weak effect (+0.3 yr) for monosomy, resulting in the pooled sample, in a non-significant difference of +1.0 yr for monosomy, and no overall effect for trisomy. For triploidy, there was a suggestive effect in both phases (+0.7 and +2.2 yr) with a significant difference of +1.3 yr in the pooled sample.

The trends for monosomy and triploidy are suggestive, particularly since other evidence suggests a paternal "origin" for a significant fraction of such events. It will be of interest to see if these trends hold up in future data from the New York series, and from spontaneous abortion data from other areas.

Finally, an unexpected suggestive paternal-age effect has been noted for balanced reciprocal rearrangements inherited from the father and detected incidentally at amniocentesis (30) (44.5 ± 8.0 yr in 11 cases vs 38.0 ± 6.0 yr in 12,038 controls. The values for maternal age were 37.2 ± 2.7 yr vs 36.3 ± 3.5 yr.) It will be of interest in subsequent data to determine if the trend persists.

CONCLUSIONS: ETIOLOGY AND MECHANISMS OF THE MATERNAL-AGE EFFECT

The evidence reviewed indicates that maternal age has a strong, ubiquitous positive association with all viable trisomies, and with most that are not viable. In contrast, there is no positive association with the one monosomy (45,X) that can be investigated. Nor is there consistent evidence for any association of paternal age with trisomy.

Indirect evidence suggests that selective effects upon fertilization, zygotes, or very early embryos may contribute to a significant proportion of the maternal-age effect for 47,+21. This evidence is based on the lack of strong maternal-age differences between cases in which the extra chromosome 21 is (a) from the mother, or (b) from the father (coupled with the apparent lack of any differential paternal-age effect in cases in which the extra chromosome 21 is patroclinous). This indirect evidence itself is somewhat inconsistent and sensitive to technical errors and possible unrecognized ascertainment biases; nevertheless, in sum it must be regarded, I believe, as making a presumptive (but not definitive) case for selection effects. Evidence from experimental animals is inconsistent and not helpful in this matter. The mechanisms for such a putative selection effect remain entirely unknown.

If selection does not account for any of the maternal-age association, then by exclusion there must be a maternal-age effect upon production of disomic ova (e.g., those with two chromosome 21's). It is usually assumed such an effect must be upon the rate of nondisjunction. Indeed, studies of factors associated with trisomy often assume they are examining variables associated with nondisjunction per se, so that case-control studies of Down syndrome may be titled "studies of nondisjunction" (see, e.g., Ref. 44). But alternative possibilities for age effects upon production of disomic ova must be considered.

First, perhaps a small fraction of ova does not begin the first meiotic division at 3 months of development, but rather much later, during adulthood, and the longer the delay the higher the rate of nondisjunction in such exceptional ova when the first meiotic divisions do occur.

Second, since most oocytes degenerate after birth, perhaps those in which disjunction occurs normally selectively die, leaving an increasing proportion in which nondisjunction will occur as age advances. (This type of relaxed selection is of course different from that discussed above, since it is only operative upon maternal gametes.)

Third, it has been postulated that normal ova have a tendency to be ovulated earlier in life than abnormal disomic ova, an implication of the complex "production-line hypothesis" (19,42).

These or similar possibilities would seem to be necessary explanations if the observations on first division maternal origin of most instances (~60%-75%) of extra chromosome 21's are correct, and if relaxed selection does not account for all of the maternal-age effect.

The mechanism of age influence upon production of disomic ova could be of 2 types: (a) an intrinsic consequence of the aging process itself in women, or (b) a response to the cumulative effects over time of environmental hazards of some type. The latter is I believe implausible, although not impossible, for the following reasons. A strong maternal-age association with trisomy 21 has been noted for over a century (16) and in all groups throughout the world which have been investigated. Moreover, even the maternal age-specific rates are apparently relatively stable in different ethnic and racial groups (see Ref. 23 and 25 for review). I believe that reported differences between groups are highly likely to be attributable to differences in ascertainment. (There is only one known exception to this of which I am aware [20], but certainly there may be others.)

Only one generally recognized environmental factor to my knowledge would appear to be sufficiently ubiquitous in space and time to account for the observations, and that is ionizing radiation. If cumulative effects of background radiation are responsible for the increase in rates in women from 0.6 per 1,000 at age 20 yr to almost 100 per 1,000 at age 49 yr--a 2-fold order of magnitude increase--or for even only 5-10% of this increase, then the rates themselves should be exquisitely sensitive to ionizing radiation. But the evidence for an association of ionizing radiation with 47,+21 is variable and inconsistent (see references in Kline, this Volume, and Ref. 21). In fact, there was a negative association with Down syndrome at Hiroshima and Nagasaki (46). A claim of an effect in Kerala in India (32), where there is high background radiation, is almost certainly due to underascertainment in controls. (See Sundaram [48], Edwards and Harnden [10], and Hook and Porter [21] for comments on this paper.) Uchida (50,51), however, does believe strongly in a radiation effect.

For the reasons above, I believe that maternal-age effects upon production of disomic ova, if any, are most likely a reflection of ubiquitous processes intrinsic to the aging process itself. I emphasize that this does not exclude the possibility that preventive intervention of some type may impede such ravages of time, nor that preventable environmental factors may influence the rate of abnormalities at all ages.

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GENETIC FACTORS INFLUENCING ANEUPLOIDY FREQUENCY

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INTRODUCTION

The presence of significant genetic or environmental risk factors for aneuploidy in the human population will almost certainly lead to heterogeneity of risk for aneuploidy among couples at risk. Such heterogeneity may be observed as a higher risk of aneuploidy among couples who have already had one aneuploid pregnancy than among those who have not. In the first section of this chapter I will discuss the existing data concerning the existence of "aneuploidy-prone" individuals or couples. I will then review a number of genetic factors which have been suggested to lead to an increased risk for human aneuploidy, examining the often conflicting evidence for such an association and possible mechanisms of action.

RECURRENCE RISK FOR HUMAN ANEUPLOIDY

Observed human chromosome abnormalities encompass several different types of anomaly: tetraploidy, monosomy X, trisomy, and structural abnormalities. The mechanisms of origin of these kinds of anomalies usually appear to be different (Ref. 31 and Hassold, this Volume), so that it seems unlikely that the same factors would predispose to more than one type of chromosome abnormality. However, such associations have been suggested by reports in the literature (23), and it would be of interest to look critically at this question. Because nontrisomic anomalies are much rarer than trisomy in livebirths, the question could best be answered by analyzing data from spontaneous abortions, in which the other types of anomaly are quite frequent. However, this chapter will usually be concerned only with risk factors for trisomy, because there are seldom sufficient data to examine other types of aneuploidy; in fact, most risk factors have been investigated only in the most common liveborn trisomy, trisomy 21.

Information about recurrence of aneuploidy could come from several sources: livebirths, pregnancies studied by amniocentesis, and spontaneous abortions. Because of the strong positive association between trisomy and maternal age, which holds for almost all possible trisomies and for spontaneous abortions as well as livebirths (22), estimates of risk must always be adjusted for the expected risk of trisomy for the maternal-age distribution involved. This is particularly important since women with trisomic pregnancies are a priori likely to be relatively old at the first trisomy, and, of course, even older in a subsequent pregnancy. It is also useful to divide the data according to maternal age to provide better risk estimates for counseling, as well as to gain insight into the possible mechanisms involved.

Trisomy in a Livebirth Following a Previous Trisomic Birth

Let us first examine the data concerning the risk of trisomy 21 in a fullterm birth for a couple with a previous trisomy 21 livebirth. The existing data have been summarized by Stene (45) and Tab. 1 shows the analysis of these data presented by Hook (25). It should be noted that while one can subdivide the cases by the maternal age at the time of the pregnancy at risk, they cannot be classified by age at the birth of the index trisomy. The data are remarkably few, but they do substantiate what is generally considered to be "common knowledge," i.e., that the risk of another trisomy is increased as much as 10-fold for pregnancies occurring in young women, but not significantly increased in older women. However, it is not possible to decide from these data whether the risk might be increased for older women whose first trisomy occurs at a relatively young age, rather than at an older age. There is also no information available in these sibships on the risk for other viable trisomies such as sex chromosome abnormalities.

Trisomy at Amniocentesis Following an Index Birth with Trisomy

More complete data on these questions have been provided by the European Collaborative Study of amniocentesis, derived from pregnancies studied by amniocentesis at about 16 weeks of gestation because of a history of a previous birth with trisomy 21 (37,46). Table 2 shows an analysis based upon the two published data sets, for which Dr. Stene kindly furnished maternal-age data permitting these calculations. Recurrence risks can now be derived for those women less than 30 yr at their trisomic birth, but 30 yr or older at the time of their amniocentesis. Data are also available for trisomies other than trisomy 21. The expected numbers of trisomy 21 were calculated from rates of trisomy at amniocentesis, using 5-yr maternal-age groups for ages below 30 yr, and single years of age above 30 yr (see caption and footnote to Tab. 2 for the source of these data). Tab. 2 indicates that when the mother was less than 30 yr at both pregnancies, the observed number of births with Down syndrome was 8-fold that expected on the basis of age alone. When a pregnancy occurred at 30 yr or older to a woman whose prior trisomy 21 child was born when she was less than 30 yr, the observed risk was only 2.5 times that expected, not significantly different from one. For women where both the index case and the amniocentesis occurred at 30 yr or above, there is no observed increase in risk over that expected.

Tab. 1. Recurrence of trisomy 21 in livebirths. Data from Stene (45) as analyzed by Hook (25).

	Maternal age for pregnancy at risk (yr)	
	<30	30+
Trisomy 21/total	3/211	1/422
Rate (%)	1.4	0.4
95% confidence limits	(0.3–4.2)	(0.06–1.70)
Approximate ratio of observed to expected	≈15	≈1

Tab. 2. Recurrence of trisomy at amniocentesis following a liveborn trisomy 21, from a European collaborative study of amniocentesis. Data from Mikkelsen and Stene (37), Stene et al. (46), and J. Stene, pers. comm.

	Maternal age at amniocentesis									
	<30 yr					≥30 yr				
	Number of pregnancies	Observed trisomy	% Trisomy	Expected trisomy*	Observed/ expected**	Number of pregnancies	Observed trisomy	% Trisomy	Expected trisomy*	Observed/ expected**
Maternal age <30 yr at index birth										
Trisomy 21	1,661	13	0.78	1.6	8.1 (3.7-12.5)	888	6	0.68	2.4	2.5 (0.5-4.5)
Other trisomy		5	0.30	1.6	3.1 (1.1-5.5)		3	0.34	1.8	1.7 (0-3.6)
Maternal age ≥30 yr at index birth										
Trisomy 21						922	7	0.76	6.7	1.0 (0.3-1.8)
Other trisomy							4	0.43	3.7	1.1 (0-2.2)

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* Expected numbers of trisomy 21 at amniocentesis were adjusted for maternal ages. For ages less than 35 yr expectations are based on livebirth frequencies presented by Hook (26), which are corrected for fetal loss after amniocentesis by multiplying by 1.43, the rate estimated by Hook (27). For maternal ages 35 yr or over, the expectations are based on rates by single year estimated by Ferguson-Smith and Yates (14) from the European amniocentesis study. For "other trisomies" the expected rates were derived from the same sources, using the relative rates of "other" trisomies to trisomy 21 for each maternal-age category.

** 95% confidence limits in parentheses.

From this data set we can also examine the question of whether a previous trisomy 21 birth increases the risk for other trisomies. The expected numbers were calculated from the relative proportions of trisomy 21 and other trisomies in each maternal-age class at amniocentesis. For mothers under 30 yr at the amniocentesis, the ratio of the observed to expected is 3.1, which is significantly greater than 1 at the 5% level. The other trisomies observed include 13, 18, XXX, XXY, and XYY. In mothers 30 yr or older at the time of the amniocentesis there was no observed increase in other trisomies over that expected on the basis of maternal age. Other types of aneuploidy did not occur frequently enough to allow detailed analysis: there were 2 nonmosaic monosomy-X pregnancies following a previous trisomy. The expected number of nonmosaic monosomy X cases in the total of 3,471 amniocenteses is 3 in 10,000, i.e., one (28).

Trisomy in Births Preceding or Following a Trisomic Spontaneous Abortion

Let us now turn to the data concerning aneuploidy which occurs in spontaneous abortions. Very scant data exist on the frequency of trisomy found in pregnancies before and after a karyotyped spontaneous abortion with aneuploidy. Table 3 shows data from retrospective studies which have examined the previous livebirths of women with a known trisomic abortion. Alberman (3) originally presented data from London which suggested a very large increase in the rate of trisomy 21 preceding a trisomic abortion. On this basis it was conjectured that the risk of a trisomic livebirth following a trisomic abortion must also be high. Data from other studies are also shown in the table, as well as data from our ongoing study in New York City. We have not confirmed Alberman's results, finding only one trisomic birth out of a total of 432 previous fullterm pregnancies to women with a trisomic spontaneous abortion. A full statistical analysis of this data would require adjustment for maternal age; we have not undertaken this in view of the small numbers available. However, our data are significantly different from the London series, and do not suggest any substantial increase in the frequency of trisomic livebirths preceding a trisomic spontaneous abortion.

Prospective data on pregnancies following a trisomic spontaneous abortion are even scarcer, as shown in Tab. 4. They include pregnancies studied at amniocentesis, as well as livebirths. Alberman (2) found 1 trisomic fetus at amniocentesis out of 73 followed pregnancies. Other studies, including our own, have found no trisomies in a total of 298 pregnancies. Obviously these data are too few to draw any firm conclusions, and would

Tab. 3. Full term births preceding a karyotyped spontaneous abortion.

Study		Karyotype of abortion				Control births
		Trisomy	Other	Normal	Unknown	
Alberman (3)	Number of births	244	232	1,042	2,472	1,072
	Number of trisomies	5	0	0	6	0
	% Trisomy	2.0	--	--	0.24	--
Boué et al. (11) Lauritsen (34)	Number of births	53	63	150		
	Number of trisomies	0	0	1		
	% Trisomy	--	--	0.67		
New York City	Number of births	432	228	1,088	3,714	3,147
	Number of trisomies	1	1	2	5	1
	% Trisomy	0.23	0.24	0.18	0.13	0.03

Tab. 4. Births or amniocenteses following a karyotyped spontaneous abortion.

Study		Karyotype of abortion			Control births
		Trisomy	Other	Normal	(≥28 weeks)
Alberman (2)	Number of births	73			
	Number of trisomies	1			
Boué et al. (11)	Number of births	117	82	106	
	Number of trisomies	0	0	0	
Lauritsen (34)	Number of births	60	49	42	
	Number of trisomies	0	0	1	
New York City	Number of births	121	87	161	181
	Number of trisomies	0	0	1	0

require adjustment for maternal age; however, they do not support an overall recurrence risk as high as the 1 in 50 originally suggested by Alberman. It should be pointed out that if the findings on recurrence of trisomy in amniocentesis apply to trisomies in spontaneous abortions as well, one would expect an increased risk only among women having a trisomy below 30 yr; few such women are likely to exist in these samples of women with a trisomic abortion, because of the strong association of a trisomic abortion with increased maternal age.

Trisomy in a Spontaneous Abortion Following a Trisomic Spontaneous Abortion

Some of the most interesting data should come from pairs of karyotyped spontaneous abortions from the same woman, since trisomy is common among spontaneous abortions, and many other trisomies besides the viable trisomies will be represented. Early analysis of such data, by the Boués and others (3,10,32,34) (Tab. 5), led to the conclusion that there was a strong tendency for trisomy to recur, since there was a much higher incidence of trisomy following a trisomic spontaneous abortion than following an abortion with a normal karyotype. This interpretation has 2 problems. First, because of the strong tendency for women who experience recurrent abortions to have abortions with normal karyotypes (10,21,47), the proportion of trisomic abortions following a chromosomally normal abortion is much smaller than that in the series as a whole, and is not the appropriate comparison group. Second, no correction was made for the fact that women having a pregnancy following a trisomic abortion will on the average be older than women having a pregnancy following an abortion with another karyotype.

Tab. 5. Karyotypes of repeated abortions from the same woman: earlier studies (3,10,32,34).

Karyotype of first abortion	n	Karyotype of second abortion			
		Normal	Trisomy	Other	% Trisomy
Normal	47	34	6	7	12.8
Trisomy	25	4	19	2	76.0
Other	15	5	6	4	40.0
Total:	87				

Tab. 6. Karyotypes of repeated abortions from the same woman: Honolulu data (Hassold, pers. comm.).

Karyotype of first abortion	n	Karyotype of second abortion			
		Normal	Trisomy	Other	% Trisomy
Normal	67	47	11	9	16.4
Trisomy	45	22	18	5	40.0
Other	30	11	9	10	30.0
Total:	142				

Tables 6 and 7 show pairs of karyotyped abortions collected from the Hawaiian series of Hassold and Jacobs (142 pairs), and our own series from New York (125 pairs). The overall frequency of trisomy among all karyotyped spontaneous abortions in both series is about 25%. If one looks simply at the overall rates of trisomy, there is an apparent deficiency of trisomies following a chromosomally normal abortion, and an apparent excess following a trisomic abortion. The proportion of trisomy following a previous trisomy is very similar in the 2 series (40% vs 44.6%). We are currently analyzing these 2 series using a linear regression model to adjust for the maternal-age effect, and, as in a previous analysis using fewer cases (51), there is no significant association between trisomy in first and second abortions once the adjustment is made for maternal age.

We can examine concordant trisomy pairs to see if there is any indication of a tendency to repeat the same trisomy. In the Hawaii and New York data 3 of the 31 concordant pairs repeated the same trisomy: 2 trisomy-16 pairs and one trisomy-2 pair. The expected number of concordant pairs based on the relative frequencies of each type of trisomy can be calculated to be 3.4, of which 2.8 should be concordant for trisomy 16, since this trisomy makes up 30% of all trisomies in abortions. There is nothing in these data to suggest that, when trisomies do recur, it is the same trisomy which tends to repeat more often than expected by chance.

The lack of evidence for a significantly increased recurrence risk for trisomy in pairs of karyotyped spontaneous abortions is not inconsistent with the livebirth data. Most women with a trisomic spontaneous abortion and a subsequent abortion tend to be over 30 yr for both pregnancies. The livebirth and amniocentesis data would predict that these older women would not show a greatly increased risk for trisomy in their second abortion.

Conclusions

The various sources of data indicate that for women who have a trisomy

Tab. 7. Karyotypes of repeated abortions from the same woman: New York City (Warburton, Stein, Kline, and Hutzler, unpubl. data).

Karyotype of first abortion	n	Karyotype of second abortion			
		Normal	Trisomy	Other	% Trisomy
Normal	82	68	7	7	8.5
Trisomy	29	12	13	4	44.6
Other	14	7	4	3	26.6
Total:	125				

21 livebirth at a relatively young age (less than 30 yr), there is a significantly increased risk for a subsequent trisomic pregnancy, especially if this second pregnancy occurs before the age of 30 yr. Although the data are few, this increased risk probably extends to other types of trisomy as well. For women who have their first trisomic birth at 30 yr. or older, there seems to be little if any increase in the risk of a subsequent trisomy beyond that predicted by the maternal age. Thus, there may be maternal age-independent causes of trisomy which are associated with an increased risk of trisomy in each pregnancy, while the maternal-age factors are not associated with such an increase in risk. For older women the maternal age-related risk apparently overwhelms any contribution to risk made by maternal age-independent factors.

The data on trisomies in spontaneous abortions are compatible with the data on livebirths, since most of the pregnancies at risk occur in women having pregnancies over the age of 30 yr. The lack of information from this source concerning repeated abortion in young women is unfortunate, since it is this sample which would be the most informative concerning whether it is trisomy in general which tends to repeat or only trisomy for a particular chromosome or group of chromosomes. Prospective and retrospective studies of women with a trisomic abortion are not yet extensive enough to provide reliable data, but later studies have not confirmed Alberman's early study suggesting a high risk of trisomy for such women. However, in the absence of sufficient data on women having a trisomic abortion at a young age, and in the light of the data on livebirths, it would still seem prudent to suggest prenatal diagnosis for young women with a spontaneous abortion known to be trisomic.

GENETIC RISK FACTORS FOR TRISOMY

Factors which increase the risk of trisomy could be inherited factors which are a permanent feature of the parental genotype, or environmental factors which persist throughout the reproductive period and act on spermatogenesis or oogenesis. Environmental factors could also act by affecting the parental genotype, e.g., exposure of a fetus in utero might lead to mitotic errors during embryogenesis, and thus to cryptic mosaicism for trisomy. Before reviewing some of the specific genetic factors which have been suggested to be related to trisomy, let us consider some general questions arising from the known epidemiology of human trisomy which should be asked whenever such a factor is postulated. In many cases the answers to these questions are not available, but it may be useful to consider what should be examined in further investigations.

(a) Is the risk factor associated with trisomy for only a particular chromosome, or group of chromosomes, or is it related to trisomy in general? If data have been collected on Down syndrome, for example, one might try to relate the factor also to other kinds of liveborn trisomies, such as sex chromosome anomalies or trisomy 18. Of course these cases may be much more difficult to ascertain in sufficient numbers. Another approach would be to use karyotyped spontaneous abortions, since almost all possible trisomies will be represented in such a series. If karyotyped spontaneous abortions are not available, one might look for an association with early spontaneous abortion in general, since 25% of such cases will be trisomic, and a large effect might be demonstrable. Comparison may be made among trisomies for chromosome groups defined by morphology or other relevant criteria. For example, if the factor being investigated is a polymorphism of a nucleolus-organizing region (NOR), found only on human acrocentric chromosomes, it would be reasonable to ask if only acrocentric trisomies are involved.

(b) How does the factor relate to parental and, particularly, maternal age? Most studies have made the implicit assumption that genetic factors related to trisomy will be most evident if one studies cases born to young women, a not unreasonable assumption given that it is only among such women that we have evidence suggesting increased recurrence risk for trisomy. Another reason for concentrating on young mothers has been the desire to identify risk factors which will permit screening of high-risk young women by prenatal diagnosis. However, there may be genetic risk factors which interact with the maternal age-related factors, whether these be operative at the time of the origin of the trisomy, or as selective factors after gametogenesis (see Hook, this Volume). Whatever the mechanism by which maternal age produces its effect, relating risk factors to particular maternal-age groups would still be valid for screening purposes.

(c) Is the risk factor found in both mothers and fathers? As described by Hassold (this Volume) many studies using chromosomal polymorphisms have estimated that about 30% of cases of trisomy 21, and probably a smaller percentage of other trisomies which could be studied, have resulted from a nondisjunctive event in the father. Any one risk factor might operate to a greater extent in one parent than the other, so that one need not find the same parental distribution of a risk factor as is found for the total set of trisomic conceptions. Evidence for the association of a given risk factor is strengthened if the parental origin of the trisomy can be determined by cytogenetic or DNA polymorphisms, and this coincides with the presence or absence of the factor in the relevant parent. One would not expect complete concordance unless the factor is very rare in couples without trisomy. Thus, if the frequency of a factor is 20% in the general population, but 40% in parents of Down syndrome patients, one is quite likely to find cases where the presence of the factor is unrelated to the syndrome, and thus is in the "wrong" parent.

(d) Does the risk factor affect the rate of production of trisomic conceptions, or merely their rate of survival? This question has already been raised by several authors with respect to the association of maternal age with trisomy frequency (Hook, this Volume), but should be considered for any factor apparently related to trisomy. At least 3/4 of all known conceptions with trisomy 21 and a much higher fraction of those with other trisomies are lost as embryonic or fetal deaths. Thus, if a particular gene is present more often in trisomy 21 livebirths than in controls, it may indicate not that the gene predisposes to trisomic conceptions, but that its presence improves the chances of such a conception surviving to term. A partial approach to the problem can be obtained by studying spontaneously aborted pregnancies with the same anomaly: if the same association is found, then selection during recognized pregnancy cannot explain the association. For example, this has been demonstrated in the case of the maternal-age effect (22). However, this cannot rule out an effect on survival in the very early period between conception and clinically recognized pregnancy, and at present it is not technically possible to study the chromosomal characteristics of such early losses.

(e) What proportion of cases can be associated with the risk factor, and what is the recurrence risk associated with the presence of the factor in the same couple or their relatives? Estimates of the relative risk can be made based on the odds ratios provided from case-control studies. For example, the data recently reported by Jackson-Cook (30) on the presence of double NORs in parents of children with Down syndrome (see below) led to an estimate of a 20-fold increase in the risk of Down syndrome for a parent with such a variant. The variant was present in 28% of parents studied, and about 1% of controls. If these figures are accurate, then it can be calculated that, from this factor alone, the overall recurrence risk of trisomy for young mothers with a Down syndrome child would be 6/1,000 (the observed figure is 8/1,000). A factor like that reported by Antanarakis (5, and see below), which was only twice as frequent in parents with Down syndrome as in controls, would contribute little to the overall raised recurrence risk for Down syndrome among young women.

RISK FACTORS RELATED TO PARENTAL CHROMOSOMAL CHARACTERISTICS

Let us now examine some specific genetic factors which have been reported to relate to the risk of Down syndrome. We shall first consider parental "chromosomal" factors which we will define as characteristics revealed by cytogenetic analysis of the individuals at risk.

Cryptic Parental Mosaicism

One possibility, first suggested by dermatoglyphic analysis of parents of children with Down syndrome (6,42), is that some parents are cryptic mosaics, with gonadal cells which are trisomic. Such mosaicism is difficult to study and impossible to rule out by any current techniques. A recent paper by Aagesen from Denmark (1), as well as some (42) but not all (19) earlier studies, finds an increased grandmaternal age on the mother's side for children with Down syndrome, and raises the possibility that gonadal mosaicism induced in the mother during her gestation in an older mother might account for this association. Parental mosaicism could only account for repeated trisomies for the same chromosome, and thus cannot account for the increased risk for different trisomies in the same couple, which is suggested by the amniocentesis data for young women.

Heteromorphisms of the Acrocentric Chromosomes

There is a very extensive literature on possible variation in morphology and behavior of the acrocentric chromosomes in parents of children with Down syndrome (reviewed by Miller [38]). These earlier studies usually suffered from small sample sizes, and failure to recognize the large amount of individual variability that normally exists among acrocentric chromosomes in the molecular structure, the morphology and staining behavior, and the transcriptional activity of the NOR region. Thus with small samples, differences could quite easily arise by chance between case and control groups. The early studies gave no consistent patterns, and there may have been selective reporting of positive studies. Much interest has been raised by a recent study by Jackson-Cook et al. (30) which reported a highly significant difference between 50 parents of children with Down syndrome and 50 controls in the incidence of a variant described as a "double NOR," and revealed by silver staining. These results were further strengthened by the finding that the parent with the double NOR was almost always the parent in which cytogenetic marker studies suggested the trisomy had originated. This would be expected if the variant were very rare in the normal population, as it seemed to be in this study. Further studies are required to confirm these results. Although trisomy 21 was the trisomy found in the offspring of the cases, the variant was not on 21 more often than on one of the other acrocentric chromosomes. Thus, interaction among all the NOR-containing chromosomes leading to increased nondisjunction has to be postulated. This leads to the prediction of an increased incidence of trisomy for all the acrocentric chromosomes, most of which will terminate as spontaneous abortions. If the relative risk is as high for all these as for liveborn trisomy 21, then one can calculate an expected increase of 2.3x the overall rate of spontaneous abortion in individuals with this variant as well, because 36% of trisomies in spontaneous abortions are for acrocentric chromosomes. However, the authors report no observed increase in spontaneous abortions in their cases. Heteromorphic variants of the acrocentric chromosomes cannot so easily be related to trisomies for the nonacrocentric chromosomes, although interchromosomal effects are possible. It will therefore be interesting to see whether any association extends to trisomy 18 and the sex chromosome trisomies, or to other trisomies found only in spontaneous abortions.

Heteromorphisms of Other Chromosomes

Heteromorphisms of other chromosomes, such as 9qh+ and Yq+, have been reported by some studies to be related to an increased risk of spontaneous abortion (12,17,39,41,50). The mechanism proposed is increased aneuploidy as the result of unusual amounts of heterochromatin or unusual pairing relationships. Again, some controlled studies of these phenomenon have produced negative results (9,17,44,49), and there is no strong evidence for an association.

Balanced Rearrangement in a Parent

Another parental chromosomal characteristic which has often been suggested to be associated with increased risk of aneuploidy is the presence of a balanced rearrangement. The abnormal pairing configurations produced by such rearrangements at meiosis have been proposed to interfere with normal meiotic segregation (Grell, this Volume), and this has been demonstrated to occur in some organisms such as Drosophila. Support for this idea in human material has been mainly from anecdotal reports of families in which trisomy or monosomy X has been found in the offspring of parents with a balanced translocation. Since the parental chromosomal studies have usually been done only because of the aneuploidy in the children, these reports obviously cannot be used to prove the validity of the hypothesis. The best data on the question come from amniocentesis in pregnancies studied because a parent is known to be a balanced translocation carrier. Table 8 shows the results of such studies in the European Collaborative Study (46) and the New York State Chromosome Registry. There is no apparent excess of trisomies among the pregnancies of translocation carriers; exact calculations of the expected number of trisomies would require adjusting for the maternal-age distribution, but the data are still too few to make this feasible. Looking at the question the other way around, one can ask if inherited translocations are found more often than expected among cases of trisomy; this would be expected if the presence of a parental translocation increased the risk of trisomy, although only a portion of the trisomic offspring might be expected to inherit the translocation. The small amount of data available are also shown in Tab. 8, and do not suggest any substantial relationship.

Tab. 8. Relationship between balanced translocations and trisomy.

Frequency of trisomy among offspring of balanced translocation carriers, from amniocentesis data	
European Collaborative Study:	1/1,096
New York State Chromosome Registry:*	0/266
Frequency of inherited balanced translocations in trisomic conceptions	
New York City spontaneous abortions:	1-2/636 (expect 1.3)
New York State Registry, trisomy 21:*	2-3/2,000 (expect 4)
New York State Registry, trisomy 21 in amniocentesis:*	0-2/470 (expect 1)

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* Data from E.B. Hook, pers. comm.

No large set of data is available on the effect of a parental supernumerary small "marker" chromosome without any phenotypic effect, and only anecdotal pedigrees exist which suggest that there might be a relationship (43a).

Increased Mitotic Aneuploidy in Parental Lymphocyte Cultures

Another parental chromosome characteristic which has received attention as a possible indicator of increased risk for aneuploidy is the rate of mitotic cells, observed usually in cultured lymphocytes, which show hyperploidy or hypoploidy. Several studies have suggested that hyperploid cells are increased in parents of children with trisomy 21 (18,44). Other studies have been concerned with the incidence of hyperploidy in couples with recurrent abortion, and several studies have suggested an increase in a low level of X-aneuploidy among such couples, particularly the women (24,44). Although this has sometimes been interpreted as real mosaicism for X-aneuploidy, the phenomenon may be more similar to that seen in aging women (20). However, there are also several reports that find no increase in observed aneuploidy above that in a control sample (29,40).

The relationship between the observed mitotic aneuploidy in the parents and the repeated abortions has been suggested to be a factor which affects both mitotic and meiotic nondisjunction at meiosis, leading to aneuploid conceptions and thus to repeated abortion of aneuploid offspring or trisomic livebirths. Those studies (44) which have found the relationship as strong in fathers as well as in mothers have hypothesized that the effect is on second division nondisjunction, which appears to be equally frequent in both sexes (Hassold, this Volume). It is also possible that the X-aneuploidy might be related to hormonal changes in women with repeated abortions, corresponding to those found in postmenopausal women. Such an effect might then be expected to result in spontaneous abortions with a normal karyotype rather than trisomy. Some light might be shed on this phenomenon if spontaneous abortions were karyotyped from women showing the increased aneuploidy. This has not been done systematically, nor have studies of the origin of the trisomy been correlated with the parent showing the mitotic aneuploidy in the cases of trisomic offspring. In our own spontaneous abortion study we karyotyped 8 abortions from women showing cells with additional X chromosomes. There were 4 normal karyotypes, 2 with monosomy X, 1 with triploidy, and 1 with trisomy. These data are obviously too few to answer the question, but do not suggest that the X-aneuploidy is related to trisomy in these women. However, this remains an intriguing phenomenon which deserves more study under scrupulously controlled conditions.

OTHER INHERITED RISK FACTORS

Let us now discuss genetic risk factors which have been suggested for aneuploidy in man which are not related to the structure or behavior of the parental chromosomes, but to the presence of particular genotypes.

Consanguinity: Recessive Mutations Increasing Nondisjunction

There have been a number of investigations which have looked for an effect of consanguinity on aneuploid frequency. These have been well-reviewed up to 1980 by Hook (25), who concluded that there was not sufficient evidence to rule out an effect of parental homozygosity for genes leading to nondisjunction (as the result of grandparental consanguinity) on the frequency of trisomy 21. Since then another study has reported an increase in trisomy 21 of about 4x among consanguineous couples in Kuwait (4), which was not accounted for by confounding with maternal age. A study in India (43) also found an increase in trisomy in uncle-niece marriages. Although Stene (reference in Ref. 44) pointed out the highly increased paternal age in this group, it seems unlikely that the paternal-age effect is large enough to explain the increase in trisomy. However, it is difficult to imagine an inbreeding effect which acts to produce trisomy when the homozygosity is in the fetus itself rather than in the parents, unless it should lead to mitotic nondisjunction, and mosaic trisomy.

Shared Parental HLA Antigens

Another factor which has been suggested to be related to the risk of trisomy is the proportion of HLA antigens shared between the parents. Mottironi et al. (38a) reported a significantly higher proportion of couples sharing two antigens among couples with a Down syndrome child than among a control sample. No particular antigen was found more often in the cases than in the controls. Ayme et al. (7) did not find a statistically significant difference among couples sharing two antigens in a similar study, although they did find a significant excess of one antigen among couples with Down syndrome children. The two studies do not really confirm one another, although there is still a significant excess of couples with shared antigens when the two series are added together. No difference was found between the frequency of shared antigens in cases with young mothers and in cases with older mothers. Some studies (8,33), but not others (35,36), have demonstrated an increased sharing of HLA antigens in couples with repeated spontaneous abortions; couples with shared antigens have even been treated by immunizing the mother to the paternal antigens (48). The suggested mechanism for such an association has usually involved a failure of the development of normal maternal immunological blocking factors during pregnancy, with subsequent fetal rejection. This should not involve the production of fetal aneuploidy as a reason for the increased spontaneous abortion. In the Mottironi study parental origin of the trisomic conception was determined in all cases with shared antigens to be maternal first meiotic nondisjunction. Thus, the mechanism for increased trisomy 21 cannot be related to an increase in origin of the trisomy, since meiosis 1 occurs before any interaction with the paternal genotype is possible. If confirmed, the association with shared HLA antigens must be related to differential survival of Down syndrome fetuses which are likely to share more antigens with the mother.

Thyroid Auto-antibodies

A long-standing, confirmed, and unexplained observation, first made by Fialkow (15), and well-reviewed by Hook (25), is that mothers of Down syndrome offspring have a higher incidence of antithyroid antibodies (TAB) than do controls. For lack of anywhere else to put this factor, it is being discussed among "genetic" traits, but the basis of the presence of the antibodies is not actually known. Recently Flannery et al. (16) examined 36 couples with children with Down syndrome and 36 control couples. Although they found no overall significant difference in the frequency of positive TAB titres (13/36 vs 7/36), they found that in the 7 cases where one parent was positive and the parental origin of the trisomy was known, the result was concordant in each case (4 mothers and 3 fathers). If these observations are confirmed, they will indicate that paternal TAB titres are as important risk factors as maternal TAB titres, implying that the presence of the antibody is related to the origin of the disomic gamete, rather than to the survival of the Down syndrome fetus in the mother. It should be noted that the previous results of Fialkow and others were even more highly significant for mothers of monosomy X offspring than for trisomic offspring, and much evidence suggests that the mechanisms causing the two different types of aneuploidy are very different. Thus, no consistent picture emerges as to the significance of the TAB association.

Alpha-1-antitrypsin Genotype

Breg et al. reviewed this subject in 1981 (13), and no new information appears to be available. An originally highly significant association between Down syndrome and the presence of particular alleles at the alpha-1-antitrypsin locus disappeared when a larger sample of data was obtained. A weak association with sex chromosome mosaicism still persisted. This is perhaps a case history worth remembering because it points out that in any condition such as Down syndrome in which so many factors are being examined, it is very likely that some statistically significant, but chance, associations will be found and reported, while negative results are likely to go unreported.

Particular DNA Polymorphisms on Chromosome 21

An association of Down syndrome with a particular haplotype on chromosome 21 as studied through the use of 4 polymorphic probes has been reported by Antanarakis et al. (5). They found one haplotype in 42% of the number 21 chromosomes found in 20 Greek Down syndrome patients, compared to 20% of controls. The association appeared to be stronger when the analysis was restricted to the 10 cases in which the chromosome participating in the nondisjunctive event could be identified. There was no significant difference between the frequency of the haplotype in all 40 parents of Down syndrome and the controls in this small sample. Confirmatory studies need to be carried out on other populations and larger samples. The genetic marker is particularly interesting because it is actually on chromosome 21, and might be associated with an increased tendency to nondisjunction. However, as the authors point out, an effect of a closely linked gene, or increased fetal survival of trisomy 21 conceptions with this genotype, are also possible ways to produce such an association.

CONCLUSIONS

Genes inducing nondisjunction are known in other organisms, but have so far eluded discovery in the human species. After so many years of study, there still exist no genetic factors which have been clearly demonstrated to be associated with an increased risk of human trisomy. Intriguing leads exist, but all require further confirmation in larger series before any one factor can be used either as a tool to screen the population for high-risk couples of young maternal age, or to shed light on the etiology of human aneuploidy.

ACKNOWLEDGEMENTS

We wish to thank Zena Stein and Jennie Kline for helpful review of the manuscript, Michelle Hutzler and Annie Pai for their analysis of the data on repeat pairs of spontaneous abortions, and Ernest Hook, Jon Stene, and Terry Hassold for permission to use unpublished data.

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ENVIRONMENTAL CAUSES OF ANEUPLOIDY: WHY SO ELUSIVE?

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INTRODUCTION

Before the development of techniques to karyotype the spontaneously aborted conceptus, studies of human aneuploidy were based on livebirths and focused almost entirely on the most common anomalies among births: abnormalities of the sex chromosomes and trisomy 21. Various studies repeatedly showed an increase in the prevalence of trisomy 21 births with maternal age, the rapid increase beginning in the mid-30s. Trisomy 13 and 18 births showed similar associations with maternal age (see Ref. 21). No other parental characteristic, including paternal age, and no environmental exposure has shown a consistent association with trisomy 21 among births.

When it became apparent that trisomy 21 livebirths comprise about 30-40% of all trisomy 21 conceptions among recognized pregnancies, it was argued that studies of spontaneously aborted conceptions (miscarriages) might yield insights into the environmental determinants of aneuploidy (26, 52). The reasons are at least two. First, since affected births are the survivors of a considerably larger cohort of affected conceptions, it seemed possible that associations with factors that influenced intrauterine survival might obscure associations with factors that influenced incidence. Second, the numbers of trisomy 21 conceptions available for study are, at least in theory, larger among recognized miscarriages than among births, with a consequent increase in statistical power to detect associations. This last argument can be extended. Trisomy 21 conceptions comprise about 12% of all trisomic conceptions among recognized pregnancies. If we assume that the causes of trisomy are not specific to particular chromosomes, then study of all autosomal trisomies, the majority of which are lethal in utero, might provide insights into the causes of trisomy 21. Two sets of observations provide circumstantial evidence that the causes of trisomy may not be specific to the particular autosome involved: (a) trisomies 13, 14, 15, 18, 20, 21, and 22 show similar associations with maternal age, and trisomy 16 is maternal-age related, although in a different manner (20,45); and (b) young women who have experienced a trisomy 21 livebirth appear to be at increased risk of a subsequent trisomic conception (54,55), and the risk does not seem to be limited to trisomy 21. Indeed, from studies of both spontaneous abortions and amniocenteses, it appears that a trisomy in one pregnancy may be associated with an elevated risk of other aneuploidies in later pregnancies (55,63).

In this chapter we review and evaluate evidence bearing on environmental causes of aneuploidy. We focus on trisomy, but also include studies of monosomy X, triploidy, and tetraploidy. Three themes are developed. First, we search for geographic differences in prevalence of anomalies. There are 4 centers that have karyotyped large, unselected series of spontaneous abortions--London, Hiroshima, Honolulu, and New York--and we begin by considering whether or not variations in the prevalence of aneuploidy between these 4 series are suggestive of either ethnic or environmental determinants of one or several forms of aneuploidy. Second, we take a critical look at epidemiologic studies that have reported connections between environmental factors and aneuploidy, searching for any single environmental exposure that has been consistently associated with any form of aneuploidy among recognized conceptions. In the third, and last, section, we consider more intensively the evidence connecting cigarette smoking to trisomy, and in particular the possibility that effects vary with maternal age. Because the cigarette is implicated in several reproductive outcomes, we permitted ourselves on this last theme to speculate on which might be the biological pathways that would connect environmental exposures with aneuploidy, if indeed such associations do exist at all.

THE PREVALENCE OF ANEUPLOIDY IN RECOGNIZED SPONTANEOUS ABORTIONS

With only one exception found by Harlap (16) in Israel among orthodox Jewish women (and still to be replicated), the maternal age-specific prevalence rates of trisomy 21 among births, and among amniocenteses, are very similar across populations. This is not true for the prevalence of aneuploidy among miscarriages. In 4 series the proportions range from 30.6% to 49.2% (Tab. 1) (27). Since the vast majority of aneuploid conceptions among the so-called "recognized" pregnancies are aborted spontaneously, these differences could reflect variations in the incidence of particular anomalies between countries and, if so, might suggest environmental or

Tab. 1. Types of aneuploidy among spontaneous abortions (taken from Ref. 27).

	Location*			
	Hiroshima	London	Honolulu	New York City
Percent aneuploid	46.9	30.6	49.2	37.6
Number aneuploid	237	288	807	789
Percent among aneuploid				
Trisomy (autosomal and double)	57.0	49.7	50.1	49.3
Monosomy X	16.9	23.6	20.4	16.4
Triploidy	20.3	12.8	14.5	14.6
Other	5.9	13.9	15.0	19.8

* Data taken from the following sources: Hiroshima (41); London (14); Honolulu (19); New York City (64).

ethnic determinants of particular types of aneuploidy. On the other hand, differences in the prevalence of aneuploidy could also reflect variations in rates of fetal death and patterns of loss across gestation for both aneuploid and euploid conceptions. We estimate that perhaps 75% of all conceptions end in fetal death and that about 3/4 of these deaths may occur before implantation (33). Thus, given that epidemiologic studies of aneuploidy draw on pregnancies that have reached the postimplantation stage (and, indeed, usually at least 6-8 weeks gestational age), observations among recognized spontaneous abortions may already reflect differential patterns of intrauterine survival in the early stages between fertilization and postimplantation. We caution, therefore, that the comparisons we are about to make are based on prevalent or surviving aneuploidies, rather than on incident cases.

Figure 1 sets out the gestational age-specific prevalence rates of aneuploidy for the 4 series of spontaneous abortions. The differences between studies are most marked in the later gestations, beginning around 16 weeks. In Hiroshima and Honolulu the prevalence of aneuploidy is similar throughout the gestational ranges studied, whereas in London and New York City the rates decrease in later gestational ages. Three of the 4 series include spontaneous abortions earlier than 8 weeks gestational age, and in each of these the prevalence of aneuploidy is low compared to rates in the 8- to 12-week period.

One approach to examining whether or not variations in the overall rate of aneuploidy may be due to variations in incidence between these 4 geographic areas is to contrast the frequencies of different types of abnormality among all aneuploidies. Let us do this by ignoring, for the moment, all euploid (chromosomally normal) abortions. Table 1 also sets out these data. Note first that proportions of trisomic abortions are similar among the 4 series. Both monosomy X and triploidy show some minor differences among series, but these differences do not vary systematically with the overall rates of aneuploidy. For example, the proportions of monosomy X in Hiroshima and New York City are nearly identical. Thus, among aneuploid spontaneous abortions, we see a rather standard pattern of anomalies across sites.

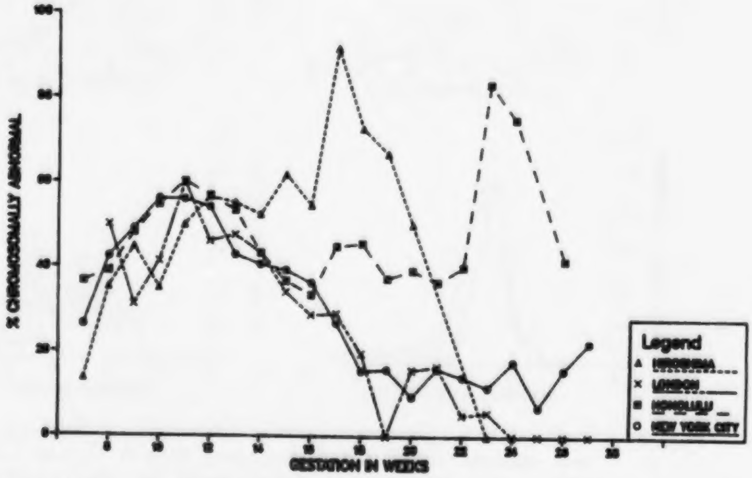

Fig. 1. Percent chromosomally abnormal (i.e., aneuploidy) by week of gestation for 4 series of karyotyped spontaneous abortions.

The similarities among series in the distributions of types of aneuploidy suggest that differences in incidence for particular aneuploidies are unlikely. This last point can be evaluated further by comparing the gestational age distributions of specific types of aneuploidy among series. Figure 2 makes these comparisons for the Hiroshima, New York City, and Honolulu series; data on the distributions of specific anomalies by single week were not solicited for the London series. Each grouping of abnormalities manifests a characteristic distribution, irrespective of the geographic location of the study. Thus, for example, in all 3 series monosomy X abortions evince a modal distribution with about 70% occurring between 11 and 14 weeks gestation; similarly, trisomies as a group are unimodal, with the peak occurring between 11 and 13 weeks gestational age. Triploidy is more uniformly distributed across the range. Given that the distributions of gestational age for each type of anomaly are so similar across sites, the data support the impression that the gestational age at abortion is primarily a function of the type of abnormality and not of the various environmental or ethnic factors that distinguish the 3 samples.

In marked contrast, for euploid abortions there are clear differences in the gestational distributions between series. (In making these comparisons, bear in mind that in Hiroshima, abortions after 20 weeks gestational age were not routinely ascertained.) Comparison of the London, Honolulu, and New York City data suggests an excess of late chromosomally normal abortions in London and New York, compared to Honolulu. For abortions of 20 weeks gestation or later the proportions are 34.6%, 24.9%, and 5.5%, respectively. The converse interpretation--an excess of early chromosomally normal abortions in Honolulu--is unlikely given the similar prevalence rates for aneuploidy in early gestations.

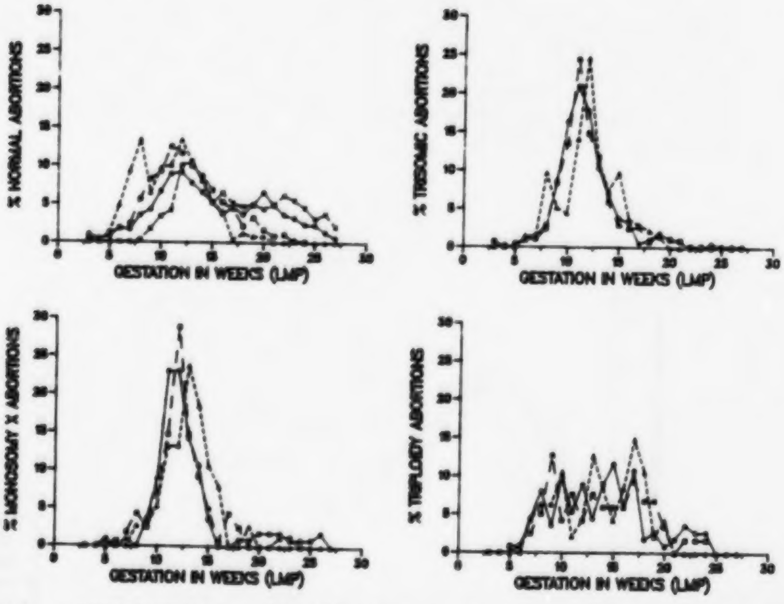

Fig. 2. Gestational age distributions of trisomy, monosomy X, triploidy, and euploid spontaneous abortions from 4 series (taken from Ref. 27). The series are represented in the graphs as follows: Hiroshima (▲); Hawaii (■); New York (●); London (x).

The data are not suggestive of differences in the incidence of different types of aneuploidy, at least in so far as recognized abortions may be considered informative. Therefore, we must for the moment accept that gross differences between countries, such as ethnicity, diet, and weather, are unlikely to influence the incidence of aneuploidy. It is true that all 4 studies are set in urban environments and thus exposures that are common across populations still remain possible influences. Since habits such as smoking, alcohol drinking, and contraceptive methods, as well as medical treatments and medication use, would be present in all, these exposures are the topic of the next section.

ENVIRONMENTAL EXPOSURES: A BRIEF SUMMARY

For the purposes of this review, the environmental exposures that have been examined in relation to aneuploidies can be grouped by the number of published reports and by the consistency of the observations. At one extreme are those environmental exposures where there is a single report, invariably of a positive association, with one or several forms of aneuploidy. In the middle are the few exposures that have been examined on many occasions, but with inconsistent results. At the other extreme, at least in theory, would be those exposures that have been examined on many occasions with consistent and convincing results. (We subsume strength of association, dose-response relations, biologic coherence, and statistical power within the summary term "convincing.") We omit from consideration observations made at the ecologic level, for example, studies of clustering of trisomy in time or place; such studies may generate hypotheses regarding possible determinants of aneuploidy, but they do not measure associations within individuals.

A variety of exposures fall in the first category of observations, and we select for comment 3 in which the timing of exposure in relation to the type of aneuploidy figures prominently in our assessment.

The first is coffee drinking. In the New York City spontaneous abortion study we observed an association between meternal coffee consumption and triploidy (28). The observation was made post hoc following a large series of exploratory analyses. In light of this fact, assertions about the statistical significance of this association await replication. The association boasts, however, of biologic coherence in at least 2 respects. First, the timing of the reported exposure (usual coffee consumption, rather than consumption during pregnancy) is compatible with the outcome observed. About 3/4 of triploid abortions are due to dispermy and the remainder are due primarily to maternal nondisjunction (23). In so far as maternal consumption of coffee "usually" is indicative of exposure around the time of conception, it is plausible that caffeine increases the likelihood of fertilization by 2 sperm. The mechanism might be either through an increased motility of sperm exposed to caffeine in the vaginal tract, or by alteration of the ability of the oocyte to prevent entry of a second sperm. We did not detect similar associations with tea or colas, but the amounts of caffeine in these beverages are less than for coffee, and thus the apparent "inconsistency" does not mitigate against effects. In further support of our observation is a clear dose-response relation.

This single observation can be contrasted with 2 others, each of which suggests that environmental exposures may not have one specific form of aneuploidy as an outcome. Watanabe (65) reports an association of the use of a variety of medications, including antipyretics, analgesics, and antibiotics, with a number of chromosomal anomalies. The timing of exposure was specified as in the perifertilization period, that is, within 3 weeks of the last menstrual period. The relative risks were 2.1 for trisomy, 5.6

for triploidy, and 14 for tetraploidy, all statistically significant and all based on small numbers of exposed and affected cases. A majority of triploid and tetraploid conceptions may take origin at the time of fertilization, and thus an association with exposures in the perifertilization period is biologically coherent. In contrast, for trisomy it has generally been assumed that the majority are owed to errors at maternal meiosis 1 and that the precipitating event would occur either in utero or during the resting stage. It is possible, however, that either the early stage of ripening of the follicle or the time around fertilization is a sensitive period. We have not discovered any biological rationale for favoring any one of the phases over the other.

The third observation is of an association between spermicide use at the time of conception, as reported by the woman, and tetraploidy and hypertriploidy. The observation, stemming from preliminary analyses in the New York City spontaneous abortion study, was compelling on biologic grounds: it is plausible that an exposure around the time of conception and after could be associated with a failure of the fertilized egg to cleave correctly at a very early stage of development. However, the finding was based on small numbers--19% of 21 abortuses with such errors exposed compared to 3.9% expected (57)--and we have failed to replicate the observation in subsequently collected data: none exposed among 30 affected pregnancies (58) compared to 5-6 expected had the original association persisted. Thus, in this instance, clear biologic plausibility has not been supported in a second test.

We move now to a second category of observations: those in which replicate studies have been made but do not yield firm conclusions.

Maternal irradiation has been examined in a systematic manner in relation to aneuploidy on at least 12 occasions. In half of the studies the maternal irradiation histories of trisomy 21 births are contrasted with those of matched normal or malformed control births. Most of the remaining studies are follow-up studies of cohorts exposed to irradiation diagnostically or therapeutically and, in one instance, to the bombings in Japan. Finally, 2 studies search for associations with aneuploidy among spontaneous abortions, and one among induced abortions. The findings of these various studies are summarized in Tab. 2.

Comparison across studies is difficult because the definitions of exposure and methods of ascertaining exposure differ. Certainly, across studies, there is no consistent evidence of a positive association between irradiation and trisomy, nor any suggestion that variations between studies correspond with variations in dose. It has been argued that the absence of effects in the Hiroshima and Nagasaki populations, where exposure is estimated to be high, casts doubt on the positive associations reported in several studies (62), especially since several of the case-control studies may suffer from differential recall. On the other hand, it has also been argued that high dose irradiation might increase both the incidence of trisomy and of intrauterine death, thereby obscuring a dose-response association with trisomy in studies based on births (62).

Some data, notably 2 studies by Uchida and colleagues (60,61), and one by Alberman et al. (2), were interpreted to suggest that the effects of irradiation were most marked in older women. In our view, however, the data do not provide strong support for this interpretation. In the earlier study Uchida and Curtis (60) observed that the average age of exposed mothers of Down syndrome offspring is greater than that of unexposed. They point out, however, that this disparity may result from the greater opportunity of being exposed with age. A similar line of reasoning was used in the later cohort study (61); within the limits of the small sample of trisomic offspring (N=11), however, there is no clear evidence that the relative risk associated with irradiation increased with age. Finally, for aneuploidies found among spontaneous abortions compared to births, the estimated mean dose to the gonad is raised in aneuploidies compared to controls in all age groups, beginning at age 20 years; it is only among teenage mothers, where numbers are few, that no effects were observed. Since the suggestion that the effects of irradiation might be most marked in older women has no strong support in the literature, the inconsistencies between the various studies (Tab. 2) are unlikely to reflect differences in the maternal-age distributions of the samples.

A second exposure that has been examined many times, especially recently, is exposure to contraceptive spermicides. With respect to aneuploidy, Jick et al. (25), in 1981, raised the possibility that spermicide use at the time of conception was associated with trisomic births. The finding was based on a small number of cases (3 among exposed and 1 among unexposed) and an ambiguous description of exposure, that is, filling of a prescription within 600 days of delivery. In spite of the obvious limitations of this study, it stimulated at least 7 other studies, nearly all of which focused on the possible relation of exposure around the time of conception to trisomy (mostly Down syndrome) among births. The relative risks (or odds ratios) as well as their 95% confidence intervals are set out in Tab. 3. Subsequent studies present a consistent picture of no association; that is, none detected a statistically significant association (at $p < .05$) between spermicide use and trisomy. However, since most relative risk estimates include within their boundaries large effects, no study excludes the possibility of a doubling or trebling in the risk of trisomy. No study considers the effects of duration of use. Thus, in evaluating these data, consistency across studies does not give confidence that the exposure is without modest effect.

Lastly, oral contraceptive use has been examined in relation to aneuploidies on several occasions. An association with triploidy among spontaneous abortions (8) was not repeated in subsequent studies (3,6,34). Nor do studies of spontaneous abortions or births suggest an association with trisomy (24,43,46,48), although there have been occasional reports suggesting that exposure in the perifertilization period may be related to trisomy 21 births (18).

SMOKING, TRISOMY, MATERNAL AGE

In this final section we turn to the single best-studied and most widespread human exposure: cigarette smoking. Here, we consider the relations of smoking to several reproductive outcomes, including trisomy. In spite of the widespread prevalence of cigarette smoking, the data in relation to trisomy are few. These data do provide, however, an opportunity to consider the possible difficulties in contrasting and interpreting observations based in spontaneous abortions and in births, the types of mechanisms that may underlie trisomy formation, and the likely timing of these mechanisms.

We begin by listing the associations that we would like to take within our purview.

(a) In the New York City spontaneous abortion study, cigarette smoking either before or at the time of the last menstrual period is associated with trisomy, and this association varies with maternal age (31). Among women under the age of 30 years, the proportions of both current and ex-smokers are greater among women experiencing term births (controls) than among women experiencing

Tab. 2

Tab. 2. Summary of epidemiologic studies relating irradiation to aneuploidy.

Study and location	Case vs control	Exposure	Odds ratio (95% CI)	Relative risk (95% CI)
Lunn (36) Glasgow	117 Down syndrome 117 Controls	"High gonad dose procedures"	1.1 (.6, 2.0)	
Carter et al. (10) London	51 Down syndrome 51 Malformed controls	< 4 abdominal ≥ 4 abdominal	.8 (.3, 2.3) .8 (.2, 2.8)	
Schull and Neel (49) Hiroshima and Nagasaki		5,582 exposed to bombings 9,452 not exposed to bombings		Down syndrome: .4 (.1, 1.5)
Uchida and Curtis (60) Winnipeg	81 Down syndrome 81 Cleft lip/palate 71 Neighbors	vs Cleft lip/palate: < 4 abdominal ≥ 4 abdominal vs Neighbors: < 4 abdominal ≥ 4 abdominal	1.9 (.8, 4.5) 11.8 (3.3, 41.6) .9 (.4, 2.0) 2.4 (1.0, 5.5)	
Sigler et al. (51) Cohen and Lilienfeld (11) Baltimore	216 Down syndrome 216 Controls	Diagnostic only Including fluoroscopic and therapeutic	1.1 (.7, 1.7) 2.4 (1.4, 4.0)	
Uchida et al. (61) Winnipeg		972 offspring born subsequent to abdominal X-rays 972 offspring born before exposure in women later exposed		Trisomy births: 10.0 (1.3, 78.0) Down syndrome: 8.0 (1.0, 63.8)
Marmol et al. (38) United States CPP	61 Down syndrome 224 Controls	Any abdominal/pelvic	1.1 (.6, 2.0)	

Stevenson et al. (56) Oxford		1,052 offspring to 630 women who had salpingograms vs expectations based on maternal age		Down syndrome: 1.4
Alberman et al. (1) London	465 Down syndrome 465 Malformed controls	Cumulative gonadal dose (mr): .1-1,999 2,000+	 1.4 (.9, 2.0) 3.1 (.8, 12.6)	
Alberman et al. (2) London	303 euploid SA* 103 aneuploid SA* 845 livebirths (controls)	.1-1,999 2,000+ 2,000+	Euploid/Control .9 (.6, 1.6) .5 (.1, 2.8)	Aneuploid/Control 1.6 (.6, 4.3) 5.4 (1.1, 25.8)
Boué et al. (6) Paris		X-ray exposure: 1) Medically 2) Professionally		Trisomy, monosomy, and polyploidy among SA:* 1) "No associations" 2) "No associations"
Cohen et al. (12) Baltimore	128 Down syndrome 128 Controls	Diagnostic only Including fluoroscopic and therapeutic	1.0 (.5, 1.9) .9 (.4, 1.8)	
Watanabe (65) Niigata City, Japan		Perifertilization exposure: Chest X-ray Abdominal X-ray		Aneuploidy among induced abortions: 1.2 (.4, 3.5) 2.4 (.7, 8.9)

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* Spontaneous abortions.

Tab. 3. Summary of studies relating spermicide use to aneuploidy.

Study and location	Time of exposure to spermicide	Outcome	Relative risk (95% CI)
Jick et al. (25) Seattle	Prescription within 600 days of delivery	Trisomy births	15.3 (1.6, 146.9)
Rothman (47) Massachusetts	Month before conception	Down syndrome	2.1[c] (.7, 6.3)[+]
Shapiro et al. (50) Collaborative Perinatal Project	First four lunar months of pregnancy	Down syndrome	1.8[a] (.1, 9.9)
Huggins et al. (22) Oxford Family Planning	Ever used diaphragm	Down syndrome	2.2 (.4, 11.0)
Polednak et al. (44) New York State	Within one year of LMP	Down syndrome	1.2 (ns)[+]
Mills et al. (40) Northern California	Before LMP	Aneuploid births	1.0 (.3, 3.4)
Bracken and Vita (7) New Haven	At conception	Aneuploid births	.8 (.1, 5.6)[+]
Cordero and Layde (13) Atlanta	Perifertilization period	Down syndrome	1.3[c] (.5, 3.8)[+]

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[a] Adjusted odds ratio.
[c] Corrected odds ratio.
[+] Odds ratio.

trisomic abortions. In other words, smoking may protect young women from trisomic abortions. In contrast, among older women, smoking is positively associated with trisomic abortion. Both findings hold for trisomy 16 and for potentially viable trisomies as a group.

(b) The observation in spontaneous abortions contrasts with observations in births. In the Collaborative Perinatal Project, the risk of Down syndrome was lower, in most maternal age groups, among women who smoked during pregnancy than among women who did not (30). Similarly, Cross and Hook (15) report from a case-control study that the frequency of maternal smoking within one year of conception was less common among mothers of Down syndrome offspring than among mothers of both normal and other malformed offspring (at the p = .10 level). This inverse association was evident in both young and older women. Table 4 summarizes these 3 observations. In order to facilitate comparison of the spontaneous abortion and livebirth data, it shows trisomy 21 abortions separate from other trisomies in spite of the small sample (13 trisomy 21 abortions) studied.

Tab. 4. Relation of trisomy to maternal smoking among women under 30 years and among women 30 years and over.

		Maternal age (yr)	
		<30	≥30
Collaborative Perinatal Project Down syndrome (30)	Relative risk (95% CI*)	.82 (.40, 1.67)	.36 (.16, .84)
New York City spontaneous abortions (31)	All trisomies Odds ratio (95% CI)	.51 (.32, .83)	1.42 (.88, 2.27)
	Trisomy 21 Odds ratio	.04^c (.06, 2.77)	1.11^c (.27, 4.54)
	Trisomy 16 Odds ratio	.34^c (.16, .76)	1.20^c (.52, 2.78)
Upstate New York Down syndrome (15)	Odds ratio (90% CI)	.8 (.4, 1.6)	.4 (.2, .9)

^{c} Corrected odds ratio.
* CI = confidence interval.

(c) Before considering these observations further, we note 6 possibly related associations. First, advanced age has been associated with a slowness to conceive (see Ref. 53). Second, smoking has been associated with infertility and with slowness to conceive in some series (4,32,42,53,59), but not in others (18,35). Third, in our New York City series, exsmokers do not experience delays in the time to conception. Fourth, current smoking is associated with an earlier age at menopause (4); exsmokers appear similar to women who have never smoked. Fifth, urinary estrogen excretion during the luteal, but not follicular, phase may be decreased in smokers compared to exsmokers and women who have never smoked (37). Sixth, aging during the premenopausal period is accompanied by a variety of hormonal changes (8).

Returning to the observations relating smoking to trisomy, we first consider women under 30 yr of age. Among such women, the effects of smoking on trisomic abortions and births are similar. This apparent protective effect of smoking might be owed either to processes that increase the probability of intrauterine death for trisomic conceptions prior to recognized miscarriage or to processes that influence the likelihood of trisomy formation at fertilization. These processes cannot yet be separated by direct observation. A mechanism relating to subclinical abortion is supported by the observed association between smoking and conception delay. Perhaps "slowness to conceive" reflects early, undetected losses. This conjecture is not easily reconciled with the similar associations of exsmoking and current smoking with trisomy, since it seems unlikely that exsmoking could influence processes after conception and, indeed, there is no evidence to suggest that exsmokers experience longer times to conception. Alternatively, constituents of the cigarette have been associated with the death of primordial oocytes and possibly also with the death of growing or preovulatory oocytes (39). Perhaps surviving oocytes are less liable to nondisjunction, either intrinsically or because of the slightly lower ovarian estrogen levels that might be expected among smokers, whether ex- or current, if atresia in the oocyte population leads to decrements in estrogen levels.

Among the older women, the findings from spontaneous abortions and births are in conflict: a protective effect of smoking is evident in births but not in abortions, where an adverse effect is possible. Again, here, conjectures on increased intrauterine loss are not compelling, not only because of the similar effects of ex- and current smoking, but also because viable and nonviable trisomies show similar associations. For the spontaneous abortion data on their own, one possibility is that smoking has sped up the biologic aging process, resulting in an elevated rate of trisomy. However, this mechanism is not compatible with the observations among births.

A synthesis of these sets of observations eludes us. Clearly, we have omitted from our speculation mechanisms relating to other hormones, including progesterone. Nor have we speculated on mechanisms that might vary with the age of the woman, although certainly the hormonal milieu in which the exposure takes place will differ for young and old. Moreover, the cigarette, having many components, may act in separate ways on one process or another. The associations set out above are not firm, and among the less firm is surely the association with trisomy.

CONCLUSIONS

In spite of more than a decade of work on the various aneuploidies in spontaneous abortions, no single maternal or paternal environmental exposure has been consistently implicated as a determinant. Even given the restrictions born of small samples and low statistical power, the positive findings are meager. One possibility is that environmental factors may not play a significant role in causing aneuploidy in humans. A second possibility is that observations in recognized pregnancies, even now that they include karyotyped spontaneous abortions, still represent only a small proportion of all aneuploidies. Perhaps we have failed to specify the relevant exposures, or perhaps effects are conditional on the time of exposure, which might, in fact, be limited to the few hours surrounding fertilization. Or perhaps susceptibility to environmental exposures that can produce aneuploidy is limited to a relatively small proportion of all women, and we have failed to specify those individual maternal characteristics (for example, estrogen levels and progesterone levels) that define susceptibility. These possibilities suggest only some of the directions which future epidemiologic and laboratory research may take.

ACKNOWLEDGEMENTS

Portions of the work for this chapter were funded by grants from the National Institutes of Health (1 R01 HD 08838, 1 R01 HD 12207, 1 R01 DA 02090, 1 R01 HD 15909). We thank Susie Parker for her assistance in the compilation and review of the literature.

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DISCUSSION

MAGNI: If I understand Dr. Kline's presentation, there is a significant increase of triploidy among the abortions of heavy coffee drinkers. Could caffeine induce diploid gametes? In yeast, a system which is rather remote from man, where we studied the induction of meiotic diploid spores or aneuploids, caffeine is by far the most active agent in inducing meiotic diploidy. It increases the spontaneous rate by a factor of 200 at doses which are compatible with those of a heavy coffee drinker.

HOOK: But this reference to caffeine would imply that tea would also show the same association as coffee drinking. Dr. Kline, did you find an effect with tea?

KLINE: No, tea and colas did not show the association. But they do not have as much caffeine.

HOOK: But if you have a dose-response curve you would expect at least some response.

MAGNI: The effect is not restricted only to caffeine, but is also exhibited by all the inhibitors of phosphodiesterase which increase the amount of cAMP in the cell.

MORISHIMA: We have studied caffeine in different systems, admittedly not in meiosis, but in somatic cell mitosis using human cells. We were unable to induce segregational errors of chromosomes, even at a 5 M concentration, which is quite high compared with the other substances that could induce segregational errors.

MAGNI: I agree completely that in mitosis caffeine is completely ineffective. Its effect is only in meiosis, and only for inducing diploid spores, not nondisjunction.

HOOK: Do you have any explanation as to why it is effective in meiosis and not in mitosis?

MAGNI: If we treat the mitotic cells, they are already diploid. (It is difficult to produce a tetraploid with caffeine.) In meiosis we block the second meiotic division.

WARBURTON: That is interesting because Dr. Kline and I did not find any association of caffeine with trisomy.

NEOPLASIA AND CYTOGENETIC ABNORMALITIES

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NEOPLASIA AND CYTOGENETIC ABNORMALITIES

This chapter discusses the relationship between cytogenetic abnormalities and neoplasia, especially in the context of aneuploidy in the genesis and progression of neoplastic transformation.

The possibility that neoplastic change is a consequence of an alteration in the cell's genetic material, and in particular its chromosomes, was first seriously suggested by von Hansemann in 1890 (14), although it is generally acknowledged that Theodore Boveri, in 1914 (4), was the first to clearly expound the notion that a cell's neoplastic properties were a consequence of a chromosome alteration, and that a malignant growth resulted from the clonal growth and proliferation of a single, chromosomally altered cell. It is of historic interest to note that in formulating his proposals, Boveri was familiar with the demonstrations of others of the occurrence of abnormal mitoses in tumor cells, and coupled these with his own observations on multipolar spindles, and consequent abnormal chromosome segregation and abnormal development, in dispermic sea urchin eggs. The term aneuploidy had not been introduced at that time, but it is evident that Boveri was aware of the importance of the aneuploid state as a causal factor responsible for abnormal growth.

Despite Boveri's stimulating hypothesis, meaningful attempts to analyze chromosome variation in neoplastic cells had to await the development and application in the 1950s of techniques that opened up the discipline of mammalian cytogenetics. These early studies showed that chromosome changes were frequently evident in cancer cells, and that a particular chromosome change--or abnormal chromosome--was often present in all the cells of a tumor, thus confirming its clonal origin. However, none of this early work revealed any consistent change in a given chromosome, or a given aneuploid state, that was always present in, or always associated with, a given type of neoplasm. The discovery of the Philadelphia chromosome in chronic myeloid leukemia (29) was perhaps the real initial boost leading to our understanding of the relationship between chromosome abnormality and cancer in man. This was followed by 2 major advances: first, the development of chromosome banding techniques that enabled us to identify individual chromosomes and chromosome regions in the human complement, a development that has been refined and extended over the past decade; and second, the developments in molecular biology which have led to the identification of genes involved in the processes of neoplastic transformation, and which have enabled us to define, detect, and locate specific DNA sequences in the human genome. Where, then, have these developments led us to in our understanding of the aneuploid state in the context of neoplasia? This is the question that I will address, but before doing so I wish to make 2 points.

First, I should make it clear that I do not confine my definition of aneuploidy to cover simply the addition or loss of one or more complete chromosomes in the normal diploid complement, but must include also the addition or loss of parts of chromosomes, i.e., partial aneuploidy.

Second, the distinction between cancers developing on a strongly predisposing genetic background and those which we loosely refer to as acquired, or environmentally induced, can of course never be absolute and is often an entirely arbitrary distinction, for genetic and environmental factors are closely interwoven and interacting. However, insight into the relevance of genomic alterations can in some cases be gained more readily from those well-defined neoplasms with a strong hereditary predisposition, and so I wish to consider these first and under a separate heading.

ANEUPLOIDY IN STRONGLY INHERITED CANCER PREDISPOSITIONS

Down and Klinefelter Syndromes

Two of the commonest, viable, constitutional aneuploid states in man are Klinefelter syndrome, with its additional sex chromosomes, and Down syndrome, with its additional chromosome number 21. It turns out that patients with both these inherited syndromes are at higher than normal risk for developing neoplastic disease. Klinefelter males have a much higher risk than normal males for developing breast cancer (15), but this may not be too surprising since such males are often characterized by a "female-like" development of breast tissue (cf. also the gonadoblastoma in streak gonads of individuals with abnormal gonadal development and a Y chromosome). Down syndrome patients on the other hand have a much higher than normal risk for acquiring reticuloendothelial neoplasms (16). The reasons for this increased risk in Down patients are obscure, but there is quite alot of evidence indicating that cells from Down patients are more responsive than normal to transformation by oncogenic viruses (38), and to the chromosome damaging effects of mutagens such as UV light, X-rays, bleomycin, etc. (40). The causes of this increased responsiveness to mutagens on the part of cells trisomic for chromosome 21 are unknown, and the question of whether this increased sensitivity is responsible for the increased incidence of malignant disease is also unanswered.

The increased risk of cancer in Klinefelter and Down patients, although significant, is relatively small, particularly in comparison with cancer risks in constitutional partial aneuploidies associated with certain childhood cancers, and in particular, with the partial aneuploid states associated with retinoblastoma and Wilms tumor.

Retinoblastoma (Rb)

Retinoblastoma is a relatively uncommon childhood tumor that affects perhaps 1 in around 18,000 children. It is of interest as follows:

(a) In 10% of cases it can be shown that the predisposition to Rb is inherited as a dominant trait with about 90% penetrance, and in these cases a tumor usually occurs in both eyes, i.e., is bilateral; 50% of the children of a bilaterally affected parent are themselves affected.

(b) In around 5% of Rb patients it is possible to demonstrate a heritable constitutional deletion of 13q14.

(c) In a number of sporadic Rb cases, cells from the tumor, but not normal tissues, have shown 13q14 deletions, but this is observed in only a minority of cases.

(d) The tumors can be successfully removed by surgery and radiotherapy in the majority (>85%) of cases, but survivors have a later, very high risk (∿15%) of developing other tumors, especially osteosarcomas, and particularly in those regions exposed to radiation during therapy.

Knudson (20) first proposed a simple and attractive hypothesis which would account for most of the features associated with Rb. The simple thesis was that Rb is a consequence of a 2-event phenomenon, involving firstly the inheritance of a mutant gene, followed by the acquisition of a second somatic mutation in the retinoblast. Predisposition would, therefore, be inherited as a dominant, but expression at the cell level would be a recessive if the same locus was involved. An alternative would be that 2 independent loci were involved, both being expressed in a dominant fashion. There is some debate as to whether the 2-mutation hypothesis can account for all of the facts (cf. Ref. 20 and 27), but I do not wish to enter into this debate here.

What is abundantly clear is that studies on the inheritance of the deletion of 13q14 in families with retinoblastoma, coupled with the finding of the 13q14 deletion in tumor cells in some patients with the unilateral sporadic form of the disease (Tab. 1), pointed quite strongly to the possibility that the Rb tumor develops as a consequence of a partial aneuploidy resulting in monosomy for the 13q14 chromosome region. This conclusion was reinforced when Sparkes et al. (36) showed that the Rb locus was very

Tab. 1. Aneuploidy (including partial aneuploidy) in Rb tumors (uni- and bilateral).

Total tumors	Monosomy 13	Tetrasomy 6p	Trisomy 1q	Trisomy 1p	Others*	Authors
17	2	9	11	--	✓	Gallie & Phillips (13)
10	0	7	1	3	✓	Kusnetsova et al. (24)
20	6	7	7	--	✓	Benedict et al. (2)
2	1	1	2	--	✓	Workman & Soukup (42)
49	9**	23	21	3		

* A range of abnormalities with monosomy #16 or #17 being most frequent.
** Three of the 9 were partial monosomies for 13q14.

closely linked to the locus coding for the enzyme, esterase D (ESD), and that ESD levels were 50% of normal in cells of patients with the hereditary form of Rb whether or not they had a cytologically demonstrable 13q14 deletion. Moreover, Benedict et al. (3) went on to show that in such a patient showing 50% levels of ESD in her normal cells, tumor cells had zero levels of ESD and a single chromosome 13. The implication of this finding was that these tumor cells had lost the normal chromosome 13 and were monosomic for the mutant chromosome 13 with the cryptic deletion of the ESD locus and an absent, or mutant, Rb locus.

The evidence from the cytogenetic and protein studies was confirmed and extended by Cavenee et al. (5) using 7 probes for DNA sequences on chromosome 13 that detected restriction fragment length polymorphisms. Moreover, these authors were able to show that in 3 out of 7 different retinoblastomas there had been a loss of one chromosome 13 (or a segment distal to q12) with duplication of the remaining homolog (or segment) resulting in homozygosity for a presumably mutant chromosome 13. In a fourth tumor, homozygosity for the 13q14 region was also evident, having clearly arisen as a consequence of mitotic recombination.

All these findings indicate that the development of hemizygosity or homozygosity of a mutant (or deleted) Rb locus on chromosome 13 is a major factor in the genesis of a retinoblastoma. Moreover, chromosome or segment loss in the heterozygote followed by duplication of the mutant chromosome or segment may be the commonest mechanism resulting in the expression of the recessive tumorigenic state. The mutation at the Rb locus may be a structural loss, or possibly a functional inadequacy. Evidently, the presence of a single copy of the normal or wild-type Rb+ allele is sufficient and necessary to prevent the development of a retinoblastoma. The wild-type allele therefore acts as a regulator for normal retinoblast development, or as a suppressor for neoplastic development, and it may not be irrelevant to note that human chromosome 13 is one of the chromosomes that suppresses tumorigenicity in human x hamster hybrid cells (19).

Nullisomy for a normal Rb+ allele, or monosomy or disomy for a mutant Rb- allele, is not the only cytological abnormality seen in retinoblastoma cells or derived cell lines. A number of other nonrandom aneuploidies have been reported. The most significant of these (Tab. 1) are the trisomy of 1q and the tetrasomy, usually due to an additional isochromosome, of 6p. Trisomy of 1q has been noted in a wide variety of tumors and is often considered to be a secondary chromosomal alteration associated with tumor progression. I shall return to this later.

Wilms Tumor

Wilms tumor, just like retinoblastoma, occurs in both hereditary and nonhereditary forms, and is the most common abdominal neoplasm in children, affecting about 1 in 10,000 (43). The autosomal dominant heritable form, which comprises some 1/3 of cases, is often associated with other congenital abnormalities, including aniridia, genitourinary abnormalities, and mental retardation. Some patients with sporadic aniridia are heterogeneous for a constitutional deletion at 11p13, and 1 in 3 of such individuals develop Wilms tumor (12). As in the case of retinoblastoma, it was suggested that the germ line deletion of the 11p region was the first of 2 or more steps resulting in the kidney tumor (21). Once again this suggestion was supported by the finding that tumor cells from some patients with sporadic Wilms tumors, but no aniridia and a normal chromosome constitution, contained an 11p13 deletion similar to that found constitutionally in individuals with inherited Wilms tumor (Tab. 2). The cytogenetic picture therefore exactly parallels that seen in retinoblastoma.

Tab. 2. Aneuploidy (including partial aneuploidy) in sporadic Wilms tumors.

Total tumors	Monosomy 11(p13)	Trisomy 1q	Others*	Authors
9	1	5	✓	Kaneko et al. (18,22)
14	6	4	✓	Douglass et al. (10)
11	3	2	✓	Slater et al. (35)
34	10	11		

* Includes 6 cases of monosomy #16.

About 10 months ago 4 groups of workers published their findings on the genetic constitutions of the 11p chromosome regions in normal kidney cells and Wilms tumor cells, as revealed by a range of DNA probes identifying restriction fragment length polymorphisms (11,23,30,32). The results were consistent with the similar studies on retinoblastoma. In one of the largest series (11) somatic deletions of specific genes in 11p were noted in 4 of 6 tumors. In all 4 cases the deletions were associated with duplications of the sister alleles in the homologous 11p regions, so that the tumor cells were homozygous for the nondeleted alleles as compared with heterozygosity for these alleles in the normal kidney cells.

The implication that may be derived from these findings is that the Wilms tumor allele, WT$^-$, must be located in the 11p chromosome region, and the deletion of the wild-type (suppressor or regulatory) allele, WT$^+$, and monosomy or disomy for the WT$^-$ state would appear to be necessary for the development of the tumor. In some cases the deletion is of a region that is sufficiently large to be seen cytologically; in others it is too small to be detected by the currently available DNA probes.

A deletion at 11p13 is not the only cytogenetic abnormality evident in the Wilms tumor cells, and as many as 1/3 of the tumors studied show trisomy of 1q, and many a monosomy of 16q (Tab. 2). These anomalies are again similar to those seen in retinoblastoma cells. It has been argued (28) that these frequent trisomies in these two childhood cancers, and in particular, trisomy 1q, may also play a role in the early stages of development of these tumors. The suggestion being that trisomy results in an increased expression of putative oncogenes in these trisomic chromosomes. The relevance of these associated aneuploidies in tumorigenesis will be referred to later, but it should be emphasized here that the tumorigenic loci for both Rb on 13q14 and WT on 11p13 are expressed as classical recessives, in contrast to the dominantly acting, retrovirally defined c-onc genes. In the case of WT, the recessive oncogenic allele (which may of course be a null allele) is located in the vicinity of the p-Ha ras oncogene, but, in company with many others, we find that p-Ha ras is not deleted in Wilms tumor cells. Moreover, it would appear that this gene is also not mutated, for Orkin et al. (30) report that transfection of Wilms tumor DNA into NIH 3T3 cells does not result in transformation, whereas, of course, transfection with mutated c-Has ras from other human tumors does result in transformation. These findings would imply that p-Ha ras is not involved in the neoplastic transformation resulting in Wilms tumor.

The childhood retinoblastoma and Wilms tumor may be referred to as deletion and/or deletion-duplication tumors. In the case of the hemizygosity clearly documented in some of the Rb tumors, the aneuploid state would have arisen as a single nondisjunctional event. In those instances, which comprise the majority, where homozygosity for those regions surrounding the Rb or WT loci are observed, the more probable origin is a single reciprocal exchange between homologs, which would give a 50% probability of yielding daughter cells homozygous for loci distal to the exchange. The less likely mechanism would be a double nondisjunction. Is this a common mechanism in inherited cancer predispositions? At the present time the answer to this question might seem to be no, but this is an area which needs to be explored more thoroughly. A number of studies have been reported on childhood neuroblastomas and on the dominantly inherited predisposition to medullary cancer of the thyroid, but the evidence for inherited partial monosomies in these, and other, cases is either nonexistent or insubstantial. On the other hand, there is increasing evidence for the importance of hemizygosity and homozygosity in many acquired cancers.

ANEUPLOIDY IN ACQUIRED CANCERS

The use of high resolution chromosome banding techniques and the enormous effort devoted to chromosome analysis of malignant cells and cell lines have revealed a plethora of cytogenetic changes in virtually every type of neoplasm studied (26). Some of these changes may reflect the consequences of the earlier exposure of progenitor cells to a mutagenic carcinogen, but the wide variety of chromosomal changes in neoplastic cells is a reflection of 3 further factors: (a) the not surprising degree of chromosomal instability in cells undergoing continued proliferation at the wrong time and often at the wrong place, and frequently under suboptimal conditions; (b) the evolutionary opportunity for the emergence of genomic changes favorable to continued proliferation under these conditions; and (c) the genomic tolerance afforded to a cell which has no functional requirement to yield progeny cells with specified differentiation functions, and in which all activities are subservient to those necessary for continued proliferation.

Because of the clonal nature of neoplastic growth, it is to be expected that the bulk of these chromosomal changes within any one neoplasm will be nonrandom, but nonrandomness is also observed between neoplasms and some specificity of change is evident between neoplasms of the same type. The chromosome anomalies that we see in these cells can therefore be considered to fall into the following 3 categories:

(a) Primary (causal) alterations--associated with genetic changes involved in the initial phases of neoplastic transformation.

(b) Secondary (selective) alterations--genetic changes which confer selective advantage in maintaining and enhancing the proliferative capacity of the transformed cell.

(c) Adventitious (neutral) alterations--having no direct, and minimal indirect, influence on the initiation, maintenance, and progress of the neoplastic state.

The overall picture that we would expect therefore is a sea of fluctuating genomic states overlaying any basic alterations associated with the very early stages of neoplastic transformation, as well as those secondary changes which confer some advantage to the maintenance and progression of the tumorigenic state. Moreover, we should strongly emphasize the possible irrelevancy to the in vivo natural state of many of the chromosome changes seen in in vitro cultured cell lines derived from tumors, and view the results of such studies with more than usual caution in any extrapolations from in vitro to in vivo. What kinds of changes then do we observe?

Among the primary alterations we include of course the partial monosomies associated with retinoblastoma and Wilms tumor, but there are other clear-cut primary changes in other human cancers. Foremost among these are changes involving chromosome rearrangements, rather than overt gains or losses of chromosome material, in leukemias and lymphomas (Tab. 3). A classical case here is the t(8/14), t(2/8), or t(8/23) reciprocal translocation of Burkitt lymphoma where the *c-myc* oncogene at 8q24 is transferred to, and activated by, the IgH locus on chromosome 14 (6,37), or where the Igk of Igλ complexes are transferred to a site adjacent to the c-myc gene on chromosome 8q24 (8). Another is the t(9/22) reciprocal translocation found in chronic myeloid leukemia where the *c-abl* oncogene on 9q34 is transferred near to the Igλ locus on 22q11 (7,31), to give the small Philadelphia chromosome. But there is less evidence here for the activation of the *c-abl* gene. There are other, rather less clear-cut translocations which may be primary, or at least early, events in neoplastic transformation (33,34), although few of these occur at sites of known oncogenes, and where they do there is no evidence for oncogene activation. In the case of complete or partial aneuploidies the picture is even less clear.

It transpires that every chromosome, or chromosome arm, has been reported as being present in an aneuploid state in one or another kind of tumor (Tab. 4). However, some chromosomes seem to be relatively rarely involved, e.g., chromosome 10, whereas others, e.g., chromosome 1, are involved frequently and often consistently in particular types of neoplasms. Trisomy for the long arm of chromosome 1, and particularly the region q25-q32, however, is a very rare event indeed in normal cells, but turns up consistently in a variety of tumors of cervix, ovary, breast, colon, melanomas, as well as a variety of reticuloendothelial neoplasms. This is evidently a secondary change which presumably confers some advantage on a proliferating malignant cell relative to its proliferating neighbors. Other changes in this category may include monosomy for 22q in meningioma, for 1p in neuroblastoma, and for 6q in acute lymphocytic

Tab. 3. Major specific translocations characterizing leukemias/lymphomas.

Translocation	Malignancy	onc gene
t(2;8)(p11-13;q24) t(8;14)(q24;q32) t(8;22)(q24;q11)	Burkitt lymphoma ALL-L3	c-myc (8q24)
t(8;21)(q22;q22)	ANLL-M2	c-mos (8q22)
t(9;22)(q34;q11)	CML (also ALL)	c-abl (9q34)
t(14;18)(q32;q31)	Follicular lymphoma	
t(15;17)(q24;q21)	ANLL-M3	c-fes (15q23)

Tab. 4. Some examples of not unusual aneuploid states in a variety of neoplasms.

Aberration	Chromosome	Neoplasm
Trisomies	3	CLL, lymphoma
	7	colon, lymphoma, melanoma
	8	CML, ANLL, colon, bladder
	12	lymphoma, CLL, seminoma
	17	CML
	19	CML
	21	ALL
Partial trisomies	1q	very wide variety
	5p	bladder
	6p	melanoma, retinoblastoma
	7q	melanoma
	12p	seminoma
	16q	bladder
	17q	CML
Monosomies	5	AML (secondary)
	7	AML (secondary)
	16	Wilms, retinoblastoma
	17	colon, retinoblastoma
	22	meningioma
Partial monosomies	1p	neuroblastoma, melanoma, secondary leukemia, etc.
	3p	SCC lung
	6q	ALL, melanoma, ovary, Burkitt lymphoma
	7q	ANLL
	11p	Wilms
	11q	AML
	13q	retinoblastoma
	16q	AML
	20q	polycythemia

leukemia (ALL), lymphomas, melanoma, etc. (see Tab. 4). Certain aneuploidies appear as the malignancy progresses and are clearly stage-related and, particularly, for example, in the case of some of the leukemias, offer a useful diagnostic aid for staging and typing.

A number of different workers have attempted to relate some of these more consistent aneuploidies to the presence of specific p-onc genes. However, since there are more than 22 different onc genes now allocated to various chromosomes and chromosome arms (Tab. 5), and as there are but 22 different autosomes and essentially 39 different chromosome arms in the haploid human complement, any superficial relationship is probably often meaningless.

Indeed, the fact that a p-onc gene is located in a chromosome region that is deleted is unlikely to be of significance in the context of the activity of the remaining partner onc gene. However, deletions that are closely adjacent to a known onc gene may be relevant in that they may result in an activation of that locus. Perhaps more important in the context of known onc genes are the polysomic changes that result in increasing their number, and possibly the numbers of relevant onc gene transcripts. Trisomy of chromosome 7, for example, will result in an extra copy of the *erb B* onc gene which codes for epidermal growth factor receptor (39), tri-

Tab. 5. Tumorigenic retroviruses and human equivalent p-onc genes.

v-onc gene	Virus/neoplasm and species of origin	Human p-onc gene chromosome location
abl	Abelson leukemia v. (mouse)	9 (q34)
erb A	Avian erythroblastosis v. (chick)	17 (p11-q21)
erb B	Avian erythroblastosis v. (chick)	7 (p13)
ets	Avian erythroblastosis v. (chick)	11 (q23-q24)
fes	Feline sarcoma v. (cat:chick)	15 (q25-26)
fgr	Feline sarcoma v. (cat)	
fms	Feline sarcoma v. (cat)	5 (q34)
fos	Osteosarcoma v. (mouse)	2
mos	Avian myeloblastosis v. (chick)	8 (q22)
myb	Avian myeloblastosis v. (chick)	6 (q15-q24)
myc	Avian myeloblastosis v. (chick)	8 (q24)
raf	Murine sarcoma v. (mouse:chick)	3 (p25)
Ha-ras 1	Harvey murine sarcoma v. (rat)	11 (p15)
Ki-ras 1	Kirsten murine sarcoma v. (rat)	6 (p23-q12)
Ki-ras 2	Kirsten murine sarcoma v. (rat)	12 (q24)
rel	Reticuloendothelial v. (turkey)	
ros	Avian sarcoma v. (monkey)	
sis	Simian sarcoma v. (monkey)	22 (q12-q13)
ski	Avian malignancy v. (chick)	1 (q12-qter)
src	Rous sarcoma v. (chick)	20
yes	Avian sarcoma v. (chick)	
	N-myc	2 (p23-p24)
	N-ras	1 (p31-p22)
	B-lym1	1 (p32)
	int-1	12

somy 8 an extra copy of the c-myc gene which is known to be active in proliferating cells (1), and trisomy 1q extra copies of the onc genes SK and B-lym-1, and of the nerve growth factor gene (46).

Clearly it has emerged from studies on some tumors that certain onc genes appear to be amplified many hundreds of times (Tab. 6), resulting in large, cytologically visible, homogeneously staining regions. In other cases amplified regions are present as double minute fragments and cells may contain hundreds of such paired fragment chromosomes. But we should emphasize here that amplifications of this order are most certainly secondary changes that are not necessary for the early stages of neoplasia, but which are probably important factors in tumor progression.

Tab. 6. Examples of amplified c-onc genes in human cancers.

c-myc	--- APML; neuroendocrine tumors; small cell Ca lines
c-N-myc	--- neuroblastoma; retinoblastoma
c-myb	--- AML
c-abl	--- CML; erythroleukemia
c-Ki-ras	--- neuroendocrine tumors
c-H-ras	--- various solid tumors

The discovery of human p-onc genes homologous to the v-onc genes of the slowly transforming retroviruses has provided a real breakthrough and an enormous boost to our attempts to understand the mechanisms involved in neoplastic transformation. The important role played by chromosome rearrangement and amplification in the action of at least some of these onc genes which act in a dominant fashion within the cell, and the role played by chromosome deletions in the recessively acting, and as yet undefined, oncogenic loci in inherited tumor predispositions, is unquestioned. The exact mechanisms involved in these processes are, however, still elusive. One onc gene, *c-sis*, has been shown to code for a growth factor, the platelet-derived growth factor (9,41); another, *c-erb B*, for a growth factor receptor, the epidermal growth factor receptor (39); the gene products of others are kinases that phosphorylate tyrosine residues and that alter the surface properties of cells, or are associated with the nucleus or with the microfilament systems of the cell (17) (Tab. 7). One might expect that the p-onc genes picked up by retroviruses are genes involved in potentiating cell division and proliferation which would be of advantage to the virus. However, the number of single gene-controlled steps involved in the interactions that result in initiating and maintaining a proliferating cycle must number into the hundreds, as indeed will the numbers of genes involved in specifying various differentiated states and in influencing feedback mechanisms to control cell numbers. We might therefore expect that an altered level of activity in any one of a whole range of different DNA sequences might influence the proliferative state, and that secondary chromo-

Tab. 7. Locations and functions of p-onc gene products.

p-onc gene	Chromosome	Cell location	Function
c-src c-abl c-fes	20 9 15	Plasma membrane	Tyrosine phosphokinase
c-erb B	7	Plasma membrane	Epidermal growth factor receptor (EGFR)
c-fms	5	Cytoplasm	
c-mos c-raf	8 3	Cytoplasm	?
c-sis	22	Plasma membrane secreted	Platelet-derived growth factor (PDGF)
c-Ha ras c-Ki ras N-ras	11 6,12 1	Plasma membrane	GTP binding
c-myb	6	Nucleus and/or Golgi	
c-myc c-fos	8 14	Nucleus	Proliferation Proliferation and differentiation (fetal membrane)
c-ski c-B lym 1	1 1	Nucleus (?)	

somal changes, including possible deletions at fragile sites (44,45), might involve a large number of different sites in the genome. In addition to alterations influencing the uptake and utilization of energy sources in the promotion of proliferation, we must also not forget that host defense mechanisms exist to eliminate abnormal cells, and that secondary genetic changes that diminish the efficiency of host surveillance mechanisms may also be favored in the evolving neoplastic cell population.

CONCLUSIONS

In conclusion, there now exists a substantial body of evidence showing that chromosome anomalies, including aneuploidy, play a significant role in the emergence and development of neoplasia. The nature of the chromosomal changes has in some cases served to pinpoint the genetic regions whose functions are altered in the process of transformation, and cytogenetics, in these instances, has provided a stepping stone to enable us to locate and to begin to understand the nature of the changes at the gene level. However, the number of possible genetic alterations which may be involved in the primary, and even more so in the secondary, changes associated with neoplastic transformation is large, and although many relatively specific chromosome anomalies have been noted in different tumor types, the task of understanding the relevance of these changes in molecular terms is a major one. Nevertheless, the linking together of the techniques of cytogenetics, cell biology, and molecular genetics provides a powerful approach towards our understanding of the how's and why's of cellular proliferation and differentiation, and to the manner of their perturbation in neoplasia.

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DISCUSSION

WARBURTON: What is the relationship, if any, between what happens in a cancer cell with trisomy (let's say trisomy 1q), and an embryonic cell which is constitutionally trisomic for something like 1 or 7, or one of these chromosomes that contains growth factors. I suspect that they all contain growth factors once we get to know about them. Do you think this is related to what happens to these trisomies during development?

EVANS: If we try to relate what we are seeing now somatically in terms of growth and cycling, we can pinpoint genes that, in fact, affect these processes as to what would happen in terms of development early in utero. If you have a trisomy 1q, for example, you are probably going to have unbalanced growth. If you have a mosaic for trisomy, you might get very distorted growth. You can think of many possibilities. For instance, if you look at peripheral blood cells that are not malignant, but contain a certain number of cells which are trisomic for 1q or for all of 1 (or trisomy 8), is there any particular selective advantage to those particular cells which are not normally cycling except in response to an immune reaction? I think there might be. I looked very hard at my data before I came away from the lab to see what the frequency of different aneuploidies was in our Edinburgh population. We have done about 40,000 metaphase spreads and have counted for hyperploids and hypoploids, and for which chromosomes were involved. The picture we have is not very clear, but it is interesting that the most frequent chromosome in Edinburgh, chromosome 10, which is present as trisomy in blood cells of normal people, is the chromosome that is least frequently involved in tumors.

WARBURTON: We also have the mosaicism that we see in amniotic fluid cultures for trisomies which I often think must reflect some kind of selection in our culture conditions for the odd trisomic cell that has a selective advantage. There does seem to be particular chromosomes that are particularly likely to produce clones like trisomy 2 and trisomy 20. One might wonder if those kinds of cells would be found more frequently as well.

HOOK: Is there any known association of environmentally influenced malignancies and chromosome abnormalities? Take the most obvious example: cancer of the lung produced by benzanthracene.

EVANS: Rowley has data on some leukemias which she thinks are a consequence of exposure to environmental agents. There are losses of chromosome 5 and chromosome 7. That is very debatable. The cancer of the lung story is very difficult. Whang-Peng reported a year ago that there is an onc gene in 3p that is lost, which is the wrong way around as far as I am concerned. It turns out that the involvement was in lines grown from the tumor. When Zech looked at the small cell carcinoma of the lung, she found the same deletion in some of the cell lines grown out, but did not find it in cells taken fresh from the body. Rowley looked at 19 cases of small cell carcinoma of the lung last year and did not get a single one with a 3p deletion. That is an example of what might happen in culture. There is a specific loss that occurs when you culture the cells, not when you analyze the cells directly.

HOOK: Your illustration of origin of sites of onc genes did not list any on the X or Y chromosomes. Do you think those are just chance events, or is there perhaps some specific biological significance?

EVANS: There is a ras gene in the X chromosome, but it is a pseudogene; it probably is not transcribed. The Y chromosome does not have enough DNA. All the DNA codes for a single sequence that must have something to do with sex differentiation, not with onc genes. But why the X chromosome does not have one, I do not know.

ROUNDTABLE DISCUSSION: HUMAN ASPECTS OF ANEUPLOIDY

Moderator: Ernest B. Hook

Participants: H.J. Evans, Terry J. Hassold,
and Dorothy Warburton

HOOK: One of the hopes of the sponsors was to have a formal presentation on mitotic nondisjunction in humans. But there did not seem to be enough material to warrant this. It appears more appropriate to deal with this topic in a roundtable discussion including previous speakers.

HASSOLD: I want to comment on occurrence of mosaic hyperdiploidy or hypodiploidy in individuals with repeated spontaneous abortions. We looked at parents with repeated chromosomally normal or abnormal trisomic abortuses to see if we could detect any kind of frequency of hyperdiploidy or hypodiploidy in somatic cells. We looked at a very small number of cases (I think it's 6 or 7 couples). There were 3 individuals with a small level of aneuploidy in blood cultures. In all cases, the karyotypes of the abortions turned out to be normal-normal karyotypes. So in our small study there was no indication of an association with these kinds of abnormalities and repeated aneuploid spontaneous abortions.

WARBURTON: I think that the excess hyperdiploidy, if it is really present, is probably not related to a tendency to meiotic nondisjunction, but to something else about the physiology of the woman which is related somehow to pregnancy. Now the problem with that is that there are some studies that have found this also to be true of fathers. That completely demolishes my theory, if the observations in fathers are true, because there is no way that fathers can affect the pregnancy directly except through their sperm.

EVANS: Let me distinguish between somatic aneuploidy and aneuploidy arising in the first meiotic division in man, because I think they are two rather different physiological states. If there is an intrinsic phenomenon that causes aneuploidy in somatic cells, I do not think that this will be reflected in meiotic events. Of course, an agent in the environment, even the internal environment, may cause nondisjunction at meiosis and at mitosis. For example, a woman with gout and taking colchicine or something may well show, with a high enough dose, effects in blood cells and in germ cells. The same would be true with a male. But mechanistically, I think they are two different kinds of division, and unless there is an additional factor such as colchicine that causes aneuploidy in that sense, I cannot see how the two processes are related. In the case of somatic aneuploidy for the X chromosome, which is very strongly age-dependent, 6% to 7% of the cells of women age 55 to 60 yr have these extra chromosomes. Dr. Buckton at Edinburgh has shown that there is an increased frequency at an earlier age in people with Alzheimer disease. But it is a very different kind of aneuploidy from that derived from meiotic processes. There are very unusual abnormal chromosomes with abnormal centromeres.

WARBURTON: The other group in which we have found this phenomenon is a group of patients with anorexia nervosa who had secondary amenorrhea, which reinforces my idea that somatic aneuploidy for X chromosomes has something to do with estrogens.

EVANS: With regard to the X chromosome seen in somatic aneuploidy, there are two chromatids that are very parallel. They do not show the waisted indentation that you see in the normal metacentric kind of chromosome. If you stain with orcein, for example, on normal preparations, you often see a sphere at the site of the centromere, which I think is probably the tubulin as a white blob. On the X chromosomes, which are abnormal, you do not see that. I am quite certain that these X chromosomes are what were called supernumerary, acentric fragments in the Love Canal study. But they are perfectly normal; they are not aberrations in the structural sense.

WARBURTON: I agree completely. When I looked at the pictures of chromosomes from the Love Canal study I recognized the supernumerary acentrics as these funny X chromosomes. I have always described them as out-of-phase Xs. Some look like Xs that you see in premature chromosome condensations; some of them look very long and thin as if they are behind in condensation; and some of them look short, fat, and prematurely separated as if they are ahead in condensation. But whatever they are, they are out-of-phase with the rest of the chromosomes in terms of replication.

HOOK: My recollection is that a large part of the somatic changes with age are in females. Is that correct, or are they also seen in males to the same extent?

EVANS: In females you get the loss of X which goes up very steeply with age, after age 55 yr, and gets to a very high level. Perhaps 10% of the cells will show this. In males, you see much less of a change; you see some loss of Y (not a great deal, but it is significant with increasing age). In addition, somatic aneuploidy of 21 is fairly common with increasing age. Also, 22 shows an increase. There is hyperploidy, as well as hypoploidy, for these small chromosomes.

PAI: With regard to aging and loss of X chromosomes, it would be of interest to look at Turner syndrome women who are, in a sense, postmenopausal at a much younger age, and at Down syndrome patients who have premature aging and an increased incidence of Alzheimer disease. The incidence of hyperdiploidy in these patients at older ages might well be different from the rest of the population.

EVANS: One might also ask if the X chromosome that is abnormal is the inactive X? In fact, it can be the active X that is lost as well as the inactive X.

HOOK: With Turner syndrome there is, associated with structural abnormalities, the isochromosome X and the X chromosome deletions. There is a relatively high proportion of cases who are mosiacs with structural X abnormality and normal X in one line, and the other line being just 45,X. I am not aware of any studies looking at changes with time, however. Presumably the 45,X line would increase as the structural X was lost.

MORISHIMA: It is a small series, but I have 3 patients with a 45,X/46,Xr(X) mosaicism who have been followed for 20 to 35 yr. The proportion of the XO to the X ring X has not significantly changed over this period.

HOOK: Another query is whether in the XX females, who have some cells which undergo nondisjunction to produce 47,XXX, is it only the inactive X that is presumably present in duplicate? Could a XXX somatic cell in an XX adult be formed with two active Xs through somatic nondisjunction, and, if formed, would it be viable? Would one of the active Xs then be inactivated?

WARBURTON: The finding from the marker studies is that almost all 47/46 mosaics have arisen as a meiotic nondisjunctive event. The original gamete was trisomic; the mosaicism has arisen secondarily as a result of the loss of one of the chromosomes that is in triplicate. We used to talk about mitotic nondisjunction as a cause of trisomy, but now we do not think that is the cause of most trisomy mosaicisms that we are seeing.

HOOK: But there is about 20% of trisomy with patterns consistent with second division meiotic nondisjunction which could also result from mitotic nondisjunction.

HASSOLD: We should, I think, be able to sort that out with the combination of the cytogenetic markers, and again with the RFLPs. We should be able to separate the meiotic 2 errors from the mitotic errors, assuming one has markers situated all along the chromosome so that one may detect crossovers.

WARBURTON: It is often said in the literature that trisomic lines occur more often in fibroblasts than in lymphocytes in a mosaic. In other words, if one does not find the trisomic lines in the lymphocytes, it is likely that you will find an individual to be trisomic in the fibroblasts if the patient looks clinically very much affected by a chromosomal syndrome. That has to be true because if one has found the line in the lymphocytes to begin with, one does not bother looking at the fibroblasts. So we are not going to find all those cases where there are fewer trisomic cells in the fibroblasts than in the lymphocytes.

FINLEY: Dr. Warburton, several years ago we took 6 individuals that had Down syndrome with mosaicism in lymphocytes and did skin biopsies. We found that IQ correlated better with percentage of trisomic 21 cells in the skin than in the lymphocytes. With regard to another matter, in genetic counseling, we believe that the recurrence risk after birth of an individual with Down syndrome associated with 47,+21/46 mosaicism is not increased. How does that fit in with your thought that trisomic cases with mosaicism result from first division meiotic errors?

WARBURTON: I think your interpretation could well be wrong. There are no empirical data on mosaics. One can imagine very readily that the tendency to mitotic nondisjunction in utero would be inherited, and thus tend to recur. But if it is true that the mosaics result from trisomic zygotes to begin with, then I suppose they should fall into the same counseling situation as the full trisomies. It might be important in such cases to actually do marker studies and try to establish whether, in fact, they did begin as trisomic zygotes or not.

ROBINSON: What about the 45,X/46,XX/47,XXX triple mosaic? Could we raise the same question about meiotic vs mitotic origin?

HOOK: The mosaic pattern you described could arise from mitotic nondisjunction in a XX or from the successive loss of two X chromosomes in a XXX individual. I recently ran some data from the New York State Chromosome Registry. As you know, the XXX, the 47 nonmosaic, has a very strong maternal-age association. The average age is about 30 or 31 yr in our data compared to results on controls of about 26 or 27 yr. When I looked at the 45,X/46,XX/47,XXY mosaics, I found to my suprise the same elevated maternal age as in the nonmosaic 47,XXX. These data are very heterogeneous, and not from amniocentesis, but from clinical cases studied by many different centers over the years. But this suggests that these triple mosaics result from just 2 early rounds of loss of the X chromosome.

WARBURTON: One might just mention that for mosaic trisomies, at least in spontaneous abortions, the maternal age is just as high as for nonmosaic trisomies. There is again evidence that they are in fact the same phenomena.

MITOSIS IN EUKARYOTIC CELLS: AN OVERVIEW OF CHROMOSOME DISTRIBUTION

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INTRODUCTION

The usual outcome of mitosis is 2 daughter cells with equal numbers and kinds of chromosomes (euploidy) rather than unequal numbers (aneuploidy). The more we learn about the underlying mechanisms the more remarkable this seems: the precise distribution of chromosomes depends critically upon structural disorder and instability, which would more commonly be associated with imprecision, that is, with aneuploidy. My aim is to review what is known about chromosome distribution in eukaryotes and to relate disorder and instability both to the normal outcome and to the origin of aneuploidy. The discussion is limited to orthodox mitosis and meiosis in organisms with localized kinetochores (on unorthodox mechanisms, see, e.g., Ref. 19; for broader reviews of mitosis, see Ref. 16 and 24).

MICROTUBULES, CENTROSOMES, AND THE CENTRAL SPINDLE

The structural requirements for equal chromosome distribution to the daughter cells are (a) the formation of a bipolar spindle and (b) the appropriate attachment ot chromosomes to that spindle. Long protein filaments, microtubules, are key components of the spindle. The intrinsic molecular polarity ot microtubules very likely determines the direction of action ot the mitotic motors (McIntosh, this Volume), and microtubules all but certainly are responsible for the mechanical attachment of chromosomes to the spindle (3,37). Polarized motors and the mechanical association of chromosomes with such motors make possible the directed chromosome movement that results in precise chromosome distribution.

The central spindle microtubules of animal cells arise by microtubule elongation from 2 centrosomes (reviews: Ref. 22 and 25). By providing initiation sites for microtubule assembly from tubulin subunits, each centrosome determines the location, molecular polarity, and direction of growth of one set of polar microtubules (so named because they radiate from the spindle poles, not because only they have intrinsic molecular polarity). The two sets of polar microtubules grow and interact with one another (the details are unknown) to produce a spindle with a centrosome at each pole and two overlapping arrays of microtubules of opposite molecular polarity (McIntosh, this Volume).

KINETOCHORE MICROTUBULES IN ORDERLY AND DISORDERLY ARRAYS

Chromosomes are associated with the central spindle by kinetochore microtubules that extend poleward from the kinetochore (centromere). Equal chromosome distribution results from the orderly arrangement of kinetochore microtubules illustrated in Fig. 1: at metaphase, all the kinetochore microtubules of one chromosome extend toward one spindle pole while those of its partner extend toward the opposite pole. In anaphase, the chromosomes will move to opposite poles and thus one chromosome of every pair is distributed to each daughter cell. The same basic principle applies in both mitosis and meiosis, but the number of chromatids involved differs. In mitosis (Fig. 1a), 2 sister chromatids have kinetochore microtubules extending toward opposite poles and one chromatid is distributed to each pole in anaphase. In meiosis, on the other hand, 2 chromosomes pair to produce a bivalent, and in the first meiotic division (Fig. 1b), partner half-bivalents have kinetochore microtubules extending to opposite poles so that 2 chromatids are distributed to each daughter cell in anaphase.

In sharp contrast with the order seen in metaphase and anaphase, almost every conceivable arrangement of kinetochore microtubules has been seen in prometaphase when chromosomes begin to interact with the central spindle (review: Ref. 40; new observations: Ref. 5,6,18). How order arises from this initial disorder is the chief puzzle posed by chromosome distribution: somehow, a variety of inappropriate kinetochore microtubule arrangements is transformed into the one arrangement consistent with equitable distribution. In older, still useful terminology, initial inappropriate kinetochore orientation is followed by reorientation; this was first clearly recognized by Hughes-Schrader (15).

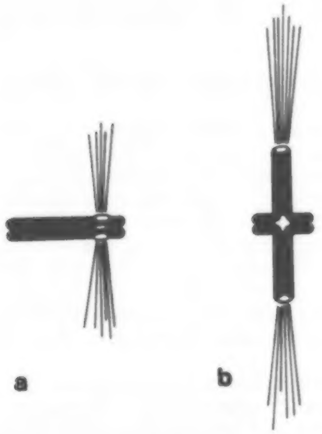

Fig. 1. The arrangement of kinetochore microtubules associated with equal chromosome distribution (a) in mitosis (and the second meiotic division) and (b) in the first meiotic division. The spindle axis is vertical, with one pole toward the top of the page and the other toward the bottom. Kinetochores are depicted as open, oval areas, the rest of the chromosome as solid black, and kinetochore microtubules as fine lines.

AN EXAMPLE OF KINETOCHORE MICROTUBULE TRANSFORMATIONS DURING REORIENTATION

We have begun to learn what happens to kinetochore microtubules during reorientation (36). Our approach is to combine micromanipulation of chromosomes in living cells with electron microscopy of those same chromosomes, permitting kinetochore microtubule arrangements to be correlated with chromosome behavior in life. Chromosomes can be detached from the spindle by micromanipulation and placed wherever desired in the cell. Invariably they reattach to the spindle and move back to it. The experimental plan is to follow the chromosome in life, wait for something interesting to happen, and then fix the cell for electron microscopy. The kinetochore microtubule arrangements observed are diagrammed in Fig. 2. A bivalent in normal orientation (Fig. 2a, right) is detached from the spindle and placed out in the cytoplasm (Fig. 2a, left); the detached bivalent lacks kinetochore microtubules (at most, only a single, short kinetochore microtubule is found before renewed chromosome movement). After only a minute or two, the detached bivalent begins to move toward the spindle equator (Fig. 2b-2c); cells fixed at this time invariably show that kinetochore microtubules have reappeared at the kinetochore of the half-bivalent facing the spindle. The new kinetochore microtubules extend toward opposite poles from one half-bivalent (Fig. 2b-2c)--a totally inappropriate arrangement for the first meiotic division (cf. Fig. 1b), which would surely lead to aneuploidy if it persisted. But it does not persist. Suddenly, the other end of the bivalent swings toward a pole, and after fixation, a single long kinetochore microtubule is seen extending in the direction of movement (Fig. 2d). As the rotation continues (Fig. 2d-2e), the kinetochore microtubule pattern necessary for equal distribution is finally established.

The most significant features of this orientation--reorientation sequence are as follows (36):

(a) By the stage shown in Fig. 2c, numerous inappropriately arranged kinetochore microtubules are present. Therefore, microtubule instability of some sort is essential: those kinetochore microtubules must either be lost or rearranged.

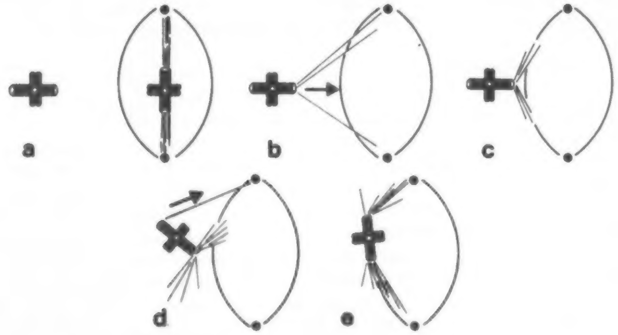

Fig. 2. Diagrams showing chromosome movement and kinetochore microtubules during the orientation and reorientation that follow bivalent detachment from the spindle by micromanipulation. Based on chromosome behavior and microtubule reconstructions in 9 grasshopper spermatocytes fixed at various times after detachment. The spindle outline is indicated and the poles are shown as solid circles; other conventions as in Fig. 1. Modified from Nicklas and Kubai (36).

(b) In this instance, chromosome movement plays a role in reorientation. The rotation shown in Fig. 2d causes the partner half-bivalents to more nearly face opposite poles, which should at least facilitate the formation of kinetochore microtubules exclusively toward one pole from each half-bivalent.

(c) Unstable anchorage of microtubules is the chief instability that permits reorientation in this example. Comparison of Figs. 2c and 2d makes this conclusion irresistible, we believe. Just envision what would be expected if the kinetochore microtubules at the right-hand half-bivalent in Fig. 2c lost their anchorage to the spindle and therefore were free to rotate clockwise along with the bivalent—a pattern like that in Fig. 2d is the result. (The microtubule patterns actually observed are more complicated but fit with the proposal, granted the expected variability in the situation of particular microtubules at the moment that rotation begins.) We argue that earlier (Fig. 2b), kinetochore microtubules must have been mechanically linked to spindle motors since force was transmitted to the chromosome and it moved toward the spindle. By the stage shown in Fig. 2d, however, the linkage of these microtubules to active motors must have lapsed--otherwise, how could these microtubules, as well as the whole chromosome, be shifted by the force transmitted by a single microtubule (at the left half-bivalent, Fig. 2d)?

INITIAL ORIENTATION IN GENERAL

The initial interaction of chromosomes with the spindle is not entirely random, despite the variety of kinetochore microtubule arrangements that results. In some chromosomes, the kinetochores are situated as shown in Fig. 1 even before kinetochore microtubules are formed: individual kinetochores are shielded by chromatin on all but one side and pairs of kinetochores face in opposite directions. Östergren (38) pointed out the potential significance of this arrangement: if an individual kinetochore tends to orient to the pole it most nearly faces, then, since its partner's kinetochore is constrained by chromosome structure to face the opposite pole, the achievement of proper orientation should be greatly facilitated. Direct evidence for preferential orientation to the pole a kinetochore faces was later provided by micromanipulation (review: Ref. 33).

Preferential orientation arises naturally given the molecular conception of the kinetochore as the unique chromosomal site for specific interactions with tubulin and/or with microtubules. Just as expected on this conception (27,34), kinetochore microtubule generation in vitro (using isolated chromosomes and tubulin) produces arrays of microtubules extending in opposite directions from the opposed kinetochores of one chromosome (e.g., Ref. 44). Note that what is in question here is the existence of interaction sites within the kinetochore whose position and polarity are constrained by chromosome structure—oppositely directed microtubule arrays could arise whether the interaction is with tubulin or with microtubules. This is important because of present uncertainty over whether kinetochore microtubules normally arise by the initiation of new microtubules, by the capture of preexisting polar microtubules, or by both (review: Ref. 40; new evidence: Ref. 5,30,31). Behind the present uncertainty may lie a complexity in kinetochore structural design and a flexibility in function that will be of exceptional interest when fully understood. Current conceptions of kinetochore molecular biology and powerful approaches for future work are described elsewhere in this Volume (see Yeh and Bloom; Brinkley et al.; and DeBrabander et al., this Volume).

Whatever the exact character of the kinetochore, constraints of chromosome structure on kinetochore position lead naturally to the formation of microtubule arrays extending generally in opposite directions. This is not, however, as great a force for perfectly arranged kinetochore microtubules as I originally thought (Ref. 34 and earlier). From the earlier light microscopic studies it was claimed that 90% of bivalents orient flawlessly at their first encounter with the spindle, but electron microscopy makes it clear that perfect arrangements of kinetochore microtubules are far rarer than that (review: Ref. 40; new evidence: Ref. 5 and 18). The probable reason for this in many cases is simply that the kinetochores are more exposed than those illustrated in Fig. 1. An extreme example is seen in male meiosis in Drosophila, where a large kinetochore protrudes from the surface of the chromosome and kinetochore microtubules extending in almost all directions are seen early in prometaphase (5,6). A complication of another sort would exist when the chromosome is bent, rather than straight, between the kinetochores of partner chromosomes, so that the 2 partner kinetochores would not face so perfectly in opposite directions as in Fig. 1b. Meiotic bivalents with several chiasmata are probable examples, but electron microscopic investigation is needed.

Thus, while kinetochore position and polarity do matter, the most frequent outcome of the initial encounter of chromosomes with the spindle is an imperfect arrangement of kinetochore microtubules.

REORIENTATION IN GENERAL

The key to understanding reorientation is Dietz' recognition (7) that it involves a difference in stability rather than some specific correction mechanism. All inappropriate orientations are unstable and soon change to some other orientation; in contrast, the one appropriate orientation is stable. Hence, reorientation occurs and recurs until, by a variety of routes, chromosomes come to the appropriate orientation and then reorient no longer.

Tension is the probable source of stability (review: Ref. 33). In the appropriate orientation, tension results from mitotic forces acting toward opposite poles (Fig. 3a), while if both partner chromosomes are oriented to the same pole, the mitotic forces are directed only to that pole and tension is absent (Fig. 3b). If the configuration shown in Fig. 3b persisted, both partners would move to the same pole and 2 aneuploid cells would result. Normally, however, reorientation begins within minutes--one partner or the other starts to move toward the opposite pole (Fig. 3d). The configuration in Fig. 3b can be stabilized by experimentally produced tension. For instance, a micromanipulation needle can be inserted in the bivalent and moved upward (Fig. 3c), stretching the bivalent and producing tension in it and in the kinetochore microtubules that anchor it to the spindle. The inappropriate orientation then is stable until the tension is released. If tension is maintained until anaphase, both chromosomes move to the same pole: nondisjunction can be induced as the experimenter directs.

We are currently studying the effects of tension and its absence by combining experiments on living cells with electron microscopy (Ault and Nicklas, unpubl. observ.). When a bivalent oriented as in Fig. 3c is fixed while under tension from a micromanipulation needle, numerous kinetochore microtubules are seen at the kinetochores of both partner chromosomes, and all of them extend toward the same pole. Each of the two kinetochore microtubule arrays (Fig. 3c) is as orderly as in an appropriately oriented bivalent (Fig. 3a). Thus, microtubule order, like orientation stability, is correlated with tension, whether the source of tension is mitotic forces or a microneedle.

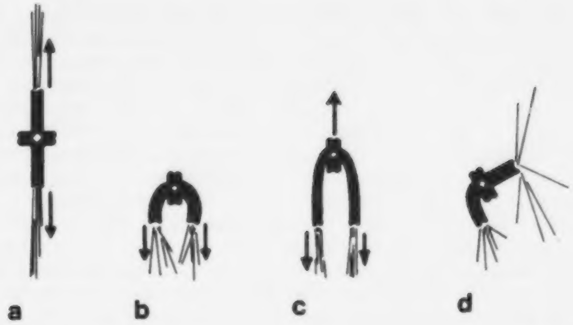

Fig. 3. Diagrams showing the effect of tension and its absence in various bivalent orientations. (a) A bivalent in stable, appropriate orientation, with spindle forces toward opposite poles (arrows) generating tension. (b) A bivalent in unstable, inappropriate orientation; both half-bivalents are oriented to the same pole and spindle forces are directed only to that pole (arrows)--tension is absent. (c) Stabilization of an inappropriate orientation by the production of tension with a micromanipulation needle (not shown) inserted in the chromosome and directed upward (upper arrow). (d) Reorientation from the configuration shown in (b). The microtubule arrangements shown are based on work in progress using grasshopper spermatocytes (Ault and Nicklas, unpubl. observ.).

An example of what happens after tension is released is diagrammed in Fig. 3d, based on observations of a cell fixed during reorientation. The kinetochore microtubule arrangement in the half-bivalent that had not reoriented (Fig. 3d, left) is far less orderly than when tension is present (Fig. 3c). The reorienting half-bivalent (Fig. 3d, right), which was moving upward when fixed, has two kinetochore microtubules extending in that direction, but also several others that extend in the general direction of the lower pole. The major (and unsolved) puzzle is the origin of the two kinetochore microtubules extending toward the upper pole. The disposition of the other microtubules is just what would be expected if the kinetochore microtubules present when the bivalent was under tension, and which then anchored it to the spindle, lost their anchorage after tension was released. This would explain their disarray and also their length. Several that are long enough to have spanned the kinetochore-to-pole distance when the bivalent was under tension no longer reach the pole (Fig. 3d, right). Our picture of reorientation here, as in the sequence considered earlier (Fig. 2c-2d), is a chromosome movement involving only 1 or 2 kinetochore microtubules; this movement is made possible by a lapse in anchorage of other microtubules, which therefore are dragged along with the moving chromosome (Fig. 3d, right).

COMMENTS AND CONCLUSIONS ON NORMAL CHROMOSOME DISTRIBUTION

Instability and the Events in Reorientation

Altering the misarranged kinetochore microtubules commonly formed when chromosomes first interact with the spindle requires that either the microtubules themselves or their anchorage to the spindle be unstable. Two general processes might therefore be expected: (a) the loss of existing kinetochore microtubules and their replacement by new ones, which sometimes extend in the appropriate direction, and (b) the persistence of existing kinetochore microtubules, but the loss of their anchorage to the spindle; this permits a microtubule rearrangement that sometimes may yield a more appropriate arrangement. Both processes are likely to contribute to reorientation and may often be related. For instance, the loss of polar anchorage, by uncapping the minus end of a kinetochore microtuble (see McIntosh, this Volume), could well promote microtubule disassembly and loss.

Sources of Instability and the Effect of Tension

We owe to Shinya Inoué and his co-workers our conception of the spindle as a labile collection of fibers in dynamic equilibrium with their subunits (review: Ref. 16). Recently, the flux of subunits into and out of spindle microtubules has been shown to be even more remarkable than previously suspected: polar spindle microtubules or their subunits appear to be completely replaced every 60-90 seconds (41; see also Ref. 42), a rate 18 times faster than in interphase microtubules (43). Equally amazing is the catastrophically rapid breakdown of some polar microtubules under conditions in which others continue to grow (28,29,30; review: Ref. 26).

So to say the least there is now ample evidence that spindle microtubules can readily be lost. The connection between this lability and the loss of kinetochore microtubules during reorientation remains uncertain, however, because kinetochore microtubules are known to be far more stable than polar microtubules (review: Ref. 40; new evidence: Ref. 30). A more immediately attractive role for the polar microtubule dynamics observed by Mitchison and Kirschner (28-30) may lie in the origin of kinetochore microtubules, as they have proposed. The continuous, rapid generation of polar microtubules at a variety of angles to the centrosomes would create repeated possibilities for their capture by kinetochores, wherever those kinetochores might be.

Turning now to unstable anchorage of kinetochore microtubules to the spindle, a simple model for the source is readily suggested (36). Suppose that at early stages kinetochore microtubules are anchored to the spindle only when engaged by active mitotic motors--force production and mechanical linkage are one and the same. Then, whenever force production ceases, e.g., when a bivalent such as the one in Fig. 3b sits immobile at one pole, its kinetochore microtubules are no longer anchored. Therefore, if 1 or 2 microtubules pointed in another direction are engaged by active motors, they can shift both the bivalent and its unanchored microtubules.

Whatever the source of instability, it must be responsive to the observed effect of tension. The model just mentioned for anchorage instability obviously links instability directly to the absence of tension, and stability to its presence: active motors stabilize anchorage because tension is generated as one set of active mitotic motors opposes the force produced by oppositely polarized motors (Fig. 3a). The stability of the microtubules themselves can also be related to tension forces, as Hill and Kirscher (13) have shown. The spindle in anaphase is capable of generating forces of exactly the magnitude that calculations suggest should stabilize microtubules (13,35). The forces necessary to stabilize orientations in prometaphase are now being measured.

Chance and Necessity in Chromosome Distribution

Reorientation is likely to have quasi-random, statistical components, as well as ones that more directly favor achievement of the appropriate orientation. A degree of randomness is suggested by studies of living cells that reveal variations in the timing and outcome of the reorientations that follow a particular initial orientation (review: Ref. 33, p. 264). An element of chance is surely likely from the microtubule dynamics just considered. Microtubules with individually unpredictable probabilities of growth or dissolution plus chance encounters of such microtubules with kinetochores and the mitotic motors will create variation upon which selection in the form of tension can act. For a concrete example, consider the origin at the stage shown in Fig. 3b of kinetochore microtubules directed upward toward the opposite pole; note that the kinetochores face away from that pole. The origin of such kinetochore microtubules requires the association of kinetochore and microtubule at an odd angle to the kinetochore: the probability of an improbable event will be heightened by the continuous generation of polar and kinetochore microtubules. Moreover, the potential importance of chance is greatly enhanced by the small numbers of kinetochore microtubules present at the critical time and by the impact of a single new kinetochore microtubule (Fig. 2d).

On this view, equal chromosome distribution results from a combination of (a) dynamics that favor stumbling on the appropriate state more or less by chance with (b) conditions that promote it more directly, such as polarized kinetochore position and, in some instances, by chromosome movement (e.g., Fig. 2d-2e).

SOME SOURCES OF ANEUPLOIDY

Not all of the many sources of abnormal chromosome numbers are included in the list that follows. My aim is more limited: to show a clear connection between some sources of aneuploidy and violations of the principles of normal chromosome distribution already considered.

(1) Failure to form a bipolar spindle. The formation of a monopolar spindle (e.g., Ref. 22 and 23) or the delayed formation of a bipolar spindle (2) results in the attachment of chromosomes to only one pole, and nondisjunction follows. In spindles with more than 2 poles, chromosomes are attached and distributed to those poles in all possible permutations (including the attachment of some individual chromatid kinetochores to 2 poles) (12,20).

(2) Absence of a functional kinetochore. Chromosomes lacking kinetochores are not attached to the spindle and for that reason, sooner or later they are lost.

(3) Absence of the usual constraints on the kinetochore position and polarity.

   (a) Chromosomes with 2 or more kinetochores commonly arise by chromosome breakage and reunion. If the kinetochores are very close together, they may act as a single large kinetochore (32; especially remarkable examples: Ref. 4; Brinkley, this Volume). Such chromosomes can be distributed normally and therefore can persist. A higher than normal rate of aneuploidy is likely, however, due to a heightened probability of interaction of a single large kinetochore with 2 spindle poles. Hence, selection for a smaller effective kinetochore is to be expected; possibilities include a reduction in copy number of kinetochore DNA sequences or inactivation of some "extra" kinetochores, giving one dominant, functional kinetochore and several inactive ones (14,45).

   If 2 kinetochores are present and are far apart ("dicentric" chromosomes), the flexibility of the intervening chromatin will no longer constrain them to act as one: a twist in that chromatin will permit them to face opposite poles. Frequent orientation of the 2 kinetochores on a single chromatid to opposite poles is the result, and chromatid breakage or aneuploidy follows (1,10).

(b) In the first meiotic division, the separation of opposed kinetochores is variable and usually they are farther apart than in mitosis (Fig. 1). If the separation is too great (or the intervening chromatin too flexible), then the kinetochores of partner bivalents will not be so well constrained to face in opposite directions as is usual. In that case, orientation errors would be expected, and are observed (47). The impact of meiotic chromosome conformation on orientation is especially interesting when more than 2 chromosomes have paired to form a multivalent (review: Ref. 39).

(c) A failure to form or maintain the normal mechanical connection between sister chromatids in mitosis or between partner half-bivalents in meiosis results in a complete absence of structural constraints on chromosome distribution. Physically separate chromosomes can neither display preferential initial orientation to opposite poles nor exert any stabilizing tension on one another. As expected, unconjoined sister chromatids in mitosis orient independently of one another and often are distributed in anaphase to the same pole rather than to opposite poles (8,9,21). The behavior of unpaired half-bivalents in meiosis is more complicated but is perfectly in accord with expectations (review: Ref. 33; new information: Ref. 46).

(4) Instability out of balance with stability. A little explored but perhaps very important source of aneuploidy stems from the delicate balance that must be struck between a spindle that is too stable for reorientation to occur and one that is so unstable that reorientation continues indefinitely. Exposure of grasshopper spermatocytes to cold temperature apparently upsets this balance. Cells returned to the normal temperature after the cold treatment have so high a frequency of reorientation that stabilization of an appropriate orientation often does not occur before another reorientation intervenes (11). Similar treatment of crane-fly spermatocytes results in maloriented chromosomes at anaphase (17). The implication that spindle stability is vulnerable to environmental insults that can result in aneuploidy clearly deserves further investigation.

CONCLUSIONS

Looking at sources of aneuploidy reinforces the view that the normally equal distribution of chromosomes in eukaryotes results from variably effective constraints of chromosome structure plus a spindle poised between chaotic change and unworkable stability. Thus, the mitotic process is naturally prone to error, though it normally works to perfection. To me it is a delight and a challenge that precise chromosome distribution seems to depend in part upon instability and chance. Such a view helps make biological sense of the molecular properties of spindle microtubules. These elements of chance and instability are modulated by the working spindle as it generates tension forces. Understanding the effect of tension on spindle structures is a major task for the future.

ACKNOWLEDGMENTS

The studies from my laboratory described here were supported in part by grant GM 13745 from the Institute of General Medical Sciences, National Institutes of Health. Special thanks are due to Bradley Smith for the drawings.

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DISCUSSION

BOND: I would like to ask a question related to the basic mechanisms underlying chromosome errors, and about how universally these are likely to apply. Where chromosomes have diffused centromeres, how do the organisms avoid the chaos you would think would result if there were a random starting point? In other words, how do the chromosomes distribute normally given a diffusion of centromeres?

NICKLAS: There certainly are, as the question implies, organisms that have chromosomes with kinetochores along their entire lengths. They have evolved several times separately, so their evolutionary situation is very interesting. Obviously they have worked very well. I assume it is because the chromosomes are so constructed that despite their having kinetochores all along their lengths, those kinetochores are held in a common relationship to one another, so that all the kinetochores, or kinetic sites, on one sister chromatid face in a direction opposite to those on the other. But, of course, we do not know. We do not even have good ultrastructural information in any of those cases.

BOND: Yeast, as you know, is an organism that is used for aneuploidy studies. How would you visualize reorientation taking place where the kinetochore is attached by just one MT?

NICKLAS: Yeast may definitely be a special case in which there is no reorientation because they get it right every time. They are almost always attached to the division apparatus. So they may become attached to opposite poles at the time of chromosome replication, or soon thereafter. That is at least a possibility, I think, though others here may know more about yeast.

RESNICK: Yeast has a very low rate of spontaneous aneuploidy, both mitotic and meiotic.

NICKLAS: It is incredible. According to the data we have been hearing, humans really do a terrible job relative to yeast. If you scale up yeast divisions from an intrinsic error rate of about one error per 10^5 cells, multiply that enough to get the number of cells in a germline of a human, and also scale up for chromosome number, the total aneuploidy rate per gamete if we were yeast would be something like 0.05%.

DE BRABANDER: When you detach a chromosome from the spindle, and you try to get it as far away as possible from the spindle (and maybe you can even try to remove it time and time again), does the cell wait to go into anaphase until all the chromosomes are back in place?

NICKLAS: Some cells would, but our cells do not always. In other words, judging from other kinds of experiments, there are cells known that would wait. But ours are not perfect. They sometimes go into anaphase with an unoriented chromosome. They always go back to the spindle (and get back to the spindle), but they are not always perfectly oriented at the time that anaphase begins.

FORD: Are homothermic animals more sensitive to spindle depolymerization because of their dependence on temperature control?

NICKLAS: The answer could well be yes.

GAULDEN: I would like to ask about the detachment of chromosomes and how they always get back on the spindle. If that occurs we would never have aneuploidy. In some work we are doing with the grasshopper neuroblast with chemical mutagens--for example, very low concentrations of benzene which induce small spindles (they reduce the size of the spindles)--we have chromosomes that become detached, come off the spindle, and never get back on the spindle. But one can be incorporated by accident into one of the nuclei (I have seen this in living cells). So this seems to be some real dysfunction of the centriole. The second point is that in one species of grasshopper we are looking at where we can see individual chromosomes and identify them, often there is one specific chromosome that comes off the spindle more often than others, which indicates that perhaps it may have a weaker attachment to the spindle. Have you had any experience of this from looking at cells with small spindles versus normal spindles?

NICKLAS: No, I have not, and that would be very interesting to do.

SPINDLE STRUCTURE AND THE MECHANISMS OF CHROMOSOME MOVEMENT

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DESIGN OF THE METAPHASE SPINDLE

Introduction

The mitotic spindle is a dynamic and transient structure; it forms to segregate the duplicate chromosomes and disappears at the completion of its task. This dynamism is complex because spindle formation includes both the polymerization of fibrous components, such as microtubules, and the organization of these components into the familiar metaphase structure. The actions of the spindle fibers on chromosomes may conveniently be divided into 2 parts: an organizational phase that precedes metaphase and a segregational phase thereafter. To discuss either of these dynamic stages with clarity, it is important to have a good view of metaphase in mind. We therefore begin our discussion of chromosome movement with a description of the metaphase spindle.

Metaphase Foci for Microtubule Ends

The spindle poles. Essentially all spindles have two specialized "poles" towards which the chromosomes will move in anaphase. These poles are regions where many spindle microtubules (MTs) terminate. In the cells of almost all animals and lower plants there is an obvious structure at each pole. For example, in mammalian cells, a normal pole contains a pair of centrioles with a halo of ill-defined, electron-dense material in which most of the MTs terminate (Fig. 1a) (49). In the cellular slime mold, *Dictyostelium discoideum*, the pole is marked by an electron-dense object with no obvious internal structure (Fig. 1b) (54), while in higher plants, the pole is rather amorphous; it is recognizable chiefly as a place where the metaphase MTs terminate, though fine structure studies have revealed an accumulation of membranous vesicles there (Fig. 1c) (34).

The structure at each well-defined spindle pole is called a "centrosome," whether or not it contains a centriole. Centrosomes are the primary organizers of MTs both in mitosis and in interphase (3,8, and De Brabander et al., this Volume). They have a replication cycle that is analogous to the chromosome cycle (17). Their duplication begins in late G_1 or early S phase, so when a cell enters mitosis it contains 2 functional centrosomes, one for each spindle pole (reviewed in Ref. 48).

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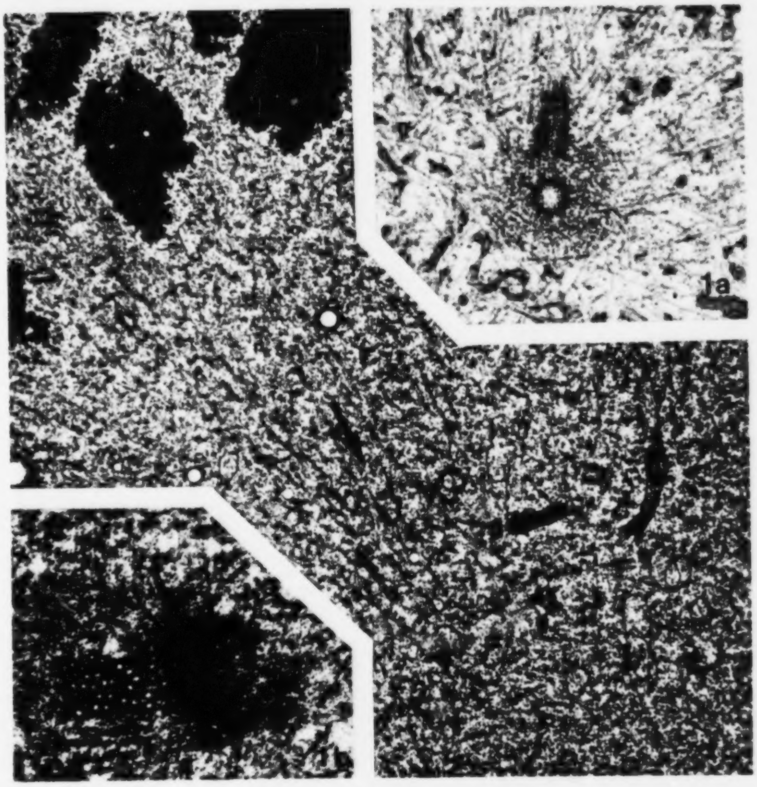


Fig. 1. Mitotic spindle poles as seen in the electron microscope. (a) From a prometaphase mammalian cell, strain PtK. Note the two centrioles and the pericentriolar material in which many MTs end. Unpublished micrograph courtesy of Kent McDonald (b) From an anaphase cell of Dictyostelium. The section is slightly oblique to the spindle axis, so on the right one sees the material of the pole and on the left one grazes into the MTs that grow from the pole. (c) From a metaphase endosperm cell of Haemanthus. Chromosomes are evident, and the arrow points toward the pole, but where the spindle ends (P), there are only a few vesicles and some granular material, no well-defined structure. Micrograph courtesy of William Jackson and the J. Cell Biol.

Mitosis in many lower eukaryotes takes place within the nuclear envelope. In all such cases, the spindle MTs end at the edge of the nucleus. There are many examples, such as Dictyostelium, where the nuclear envelope is fenestrated at the poles. The MTs focus on these breaks, as if a cytoplasmic influence helped to form a spindle in the nucleoplasm. In other cases, such as Saccharomyces, there is a specialized, spindle-pole structure built right into the nuclear envelope. This structure behaves in both interphase and mitosis like a conventional centrosome (11). In some fungi the spindle is intranuclear and there is neither a visible break nor a specialization in the nuclear envelope. Usually the ends of the spindle lie just opposite a pair of normal-looking centrosomes that is situated in the cytoplasm immediately beside the nucleus. In these cases the mode of communication between nucleus and cytoplasm is mysterious (27,28).

The chromosomes. Each mitotic metaphase chromosome possesses 2 sites of spindle fiber attachment at its centromere. These "kinetochores" are another significant focus of MT ends. In yeasts (63) and *Dictyostelium* (54) only 1 or 2 MTs end at each kinetochore (Fig. 2a), while at the kinetochores of the higher plant, *Haemanthus*, there are as many as 175 (6) (Fig. 2b). Kinetochores of the rat kangaroo mark the ends of 20-45 MTs (49) (Fig. 2c), and the green alga, *Oedogonium*, is about the same (Fig. 2d). The number of MTs ending on each kinetochore seems to depend on chromosome size, but has no effect on chromosome velocity (57). There is no known effect of kinetochore microtubule number on the fidelity of chromosome segregation, but this point may merit investigation.

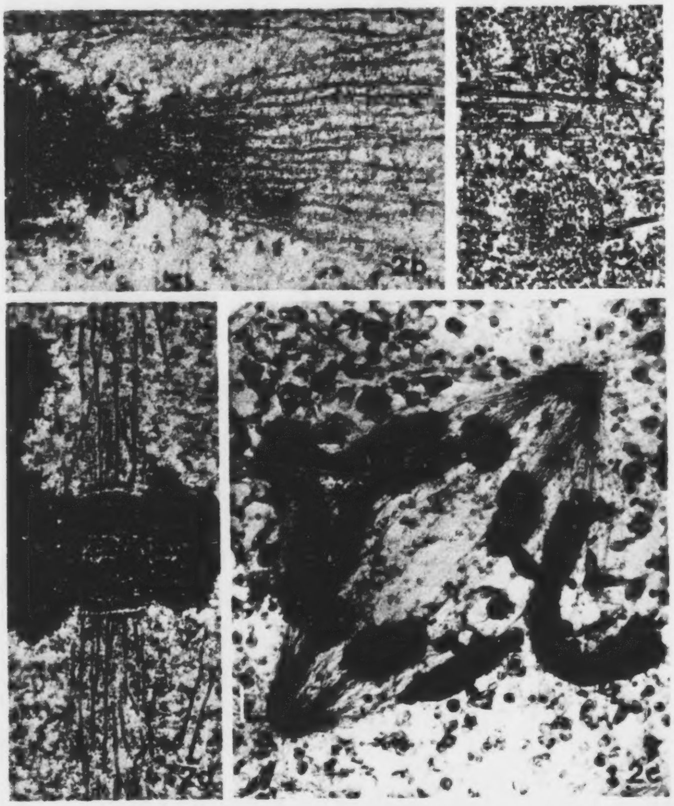
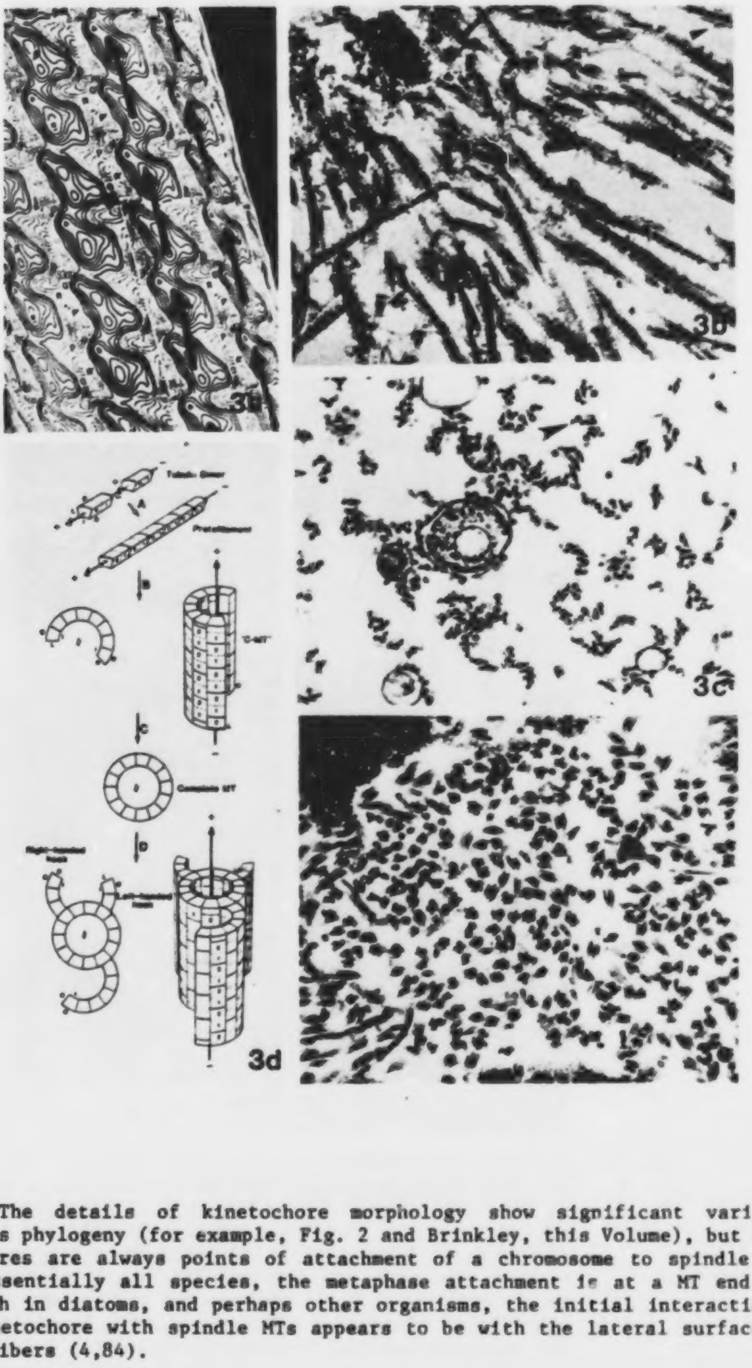


Fig. 2. Electron micrograph of mitotic kinetochores. (a) Two chromosomes (C) from *Dictyostelium*. The upper one shows a clear kinetochore on the right with a MT ending in a density just to the right of the plates that abut the chromatin (unpubl. micrograph courtesy of Urs-Peter Roos). (b) A kinetochore from *Haemanthus*. Many MTs end in the blob of chromatin (unpubl. micrograph courtesy of Peter Hepler). (c) An anaphase PtK cell lysed with triton in an equilibrium mixture of tubulin and MTs, then thick sectioned for the high voltage EM. Bundles of MTs ending on chromosomes, i.e., the kinetochore fibers, are evident. (d) A metaphase chromosome from *Oedogonium* showing numerous MTs ending on each of the sister kinetochores (unpubl. micrograph courtesy of Matthew Schibler).



The details of kinetochore morphology show significant variation across phylogeny (for example, Fig. 2 and Brinkley, this Volume), but kinetochores are always points of attachment of a chromosome to spindle MTs. In essentially all species, the metaphase attachment is at a MT end, although in diatoms, and perhaps other organisms, the initial interaction of a kinetochore with spindle MTs appears to be with the lateral surfaces of the fibers (4,84).

←

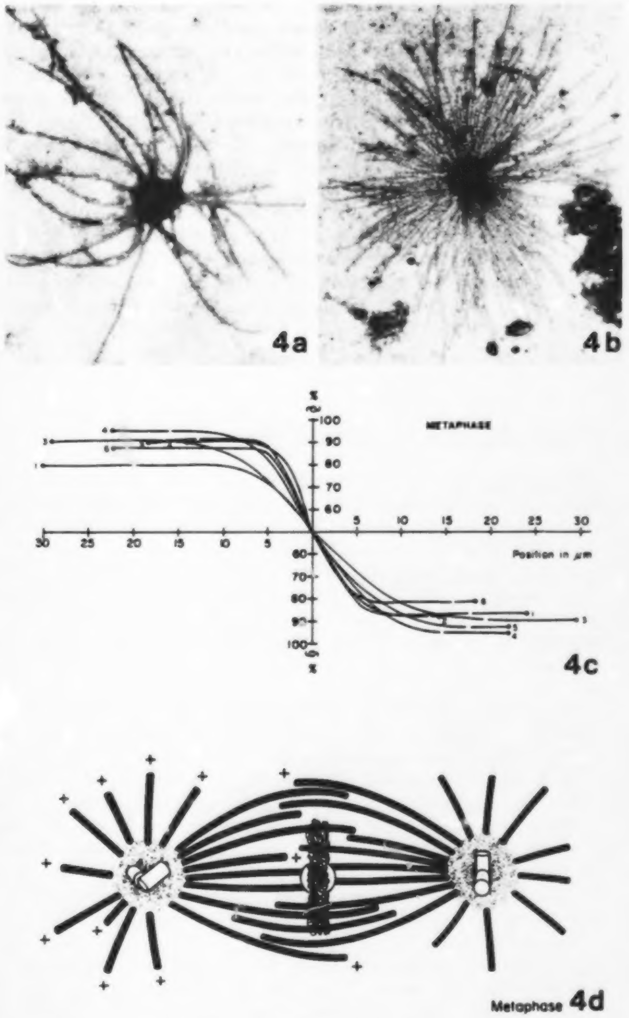
Fig. 3. The structural polarity of microtubules. (a) A drawing of the contours of electron density visible in negatively stained sheets of tubulin, arranged and folded to represent a tubule. The arrows depict the parallel arrangement of each of the heterodimers of tubulin in the MT wall. Redrawn from Baker and Amos. (b) and (c) Metaphase spindles lysed in dynein under conditions that promote dynein-binding to MTs. Both micrographs show an asymmetric arrangement of the dynein (arrow heads). In (b) many dynein arms are tipped toward the spindle pole at the upper left, and in (c) essentially every MT cross-section shows an arm with a small protrusion pointing clockwise. Both micrographs are courtesy of Leah Haimo. (d) The pathway of formation of the tubulin hooks and the way in which they reveal MT polarity. Redrawn from Heidemann and McIntosh. (e) A cross-section from a metaphase PtK cell lysed in tubulin under hook-forming conditions. Courtesy of Ursula Euteneur.

Structural Polarity of Spindle MTs

The polarity of MTs in general. MTs possess a structural polarity as a result of their assembly by the head-to-tail polymerization of heterodimers. This polarity can be visualized in flagellar MTs with high-resolution electron microscopy (3), but pictures of other MTs taken thus far have lacked the space resolution to display the polarity which chemical studies tell us must be present. From the structure of heteromorphic assemblies of cytoplasmic tubulin (7), however, one can deduce a likely model for the morphology of a spindle MT (Fig. 3a). In such models, structural polarity is evident.

Since the polarity of spindle MTs is not visible by conventional microscopic methods, indirect probes for MT orientation have been sought. The first criterion used to infer MT polarity was the site of polymer initiation. Early studies of spindle formation with both light and electron microscopy suggested that spindle poles nucleated MT assembly (reviewed in Ref. 32). It was therefore assumed that all the MTs emanating from a pole were oriented alike relative to the centrosome (51). Some experimental evidence suggested that the kinetochores were also sites of MT initiation in vivo (31,62), so it was further assumed that the MTs attached to kinetochores were oriented in the metaphase spindle antiparalleled to the MTs that emanated from the poles (51). The first real assay for MT polarity was the rate at which subunits would add to a given MT end. MT assembly is asymmetric in the sense that one end tends to add and lose subunits faster than the other (2). Kinetic analysis of MTs grown from centrosomes and kinetochores in vitro confirmed the polarity assignments made on the basis of the presumed sites of polymer initiation in vivo (8,80).

Two structural methods for displaying MT polarity are now available: (a) The flagellar enzyme dynein will bind in vitro to all kinds of MTs. Under the right conditions, asymmetries in the morphology of the dynein-MT complex reveal the polar orientation of the decorated MT (81) (Fig. 3b and c). (b) Tubulin itself can be polymerized under nonphysiological conditions to form hook-shaped sheets on the surfaces of existing MTs (29) (Fig. 3d). When these hooks are viewed in cross-section, the direction they curve (clockwise or counterclockwise) defines which way one is looking at the MT they decorate (Fig. 3d and e). The molecular features of the MT surface that allow these two structural probes to work are not yet understood, but both methods are now well tested. They clearly reveal the polarity of the decorated MT (25,50).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4c
Metaphase 4d

Fig. 4. The polarity of spindle microtubules. (a) and (b) Centrosomes from CHO cells with associated MTs initiated in vitro. The uniformity of MT lengths in the two micrographs suggests that all MTs emanating from a centrosome are of the same polarity. The difference in MT number reflects the change in the MT-initiating capacity of a centrosome as it goes from interphase (a) to mitosis (b). Micrographs courtesy of Ryoko Kuriyama and the *J. Cell Biol.* (c) The fraction of MTs with counterclockwise vs clockwise curving hooks as a function of position along the spindle axis in metaphase cells of *Haemanthus*. Courtesy of the *J. Cell Biol.* (d) A diagram depicting the polarity of spindle MTs in the metaphase cell.

Polarity assignments based on MT growth kinetics (8,80) and on both structural methods show that essentially all of the MTs emanating from interphase (Fig. 4a) or mitotic (Fig. 4b) centrosomes are of the same polarity: the fast-growing or "plus" end is distal to the pole. The growth kinetic method is not, however, suitable for studying spindle MTs in situ, so more detailed analysis of spindle MT polarity has been done only with the direct structural approaches. Analysis with the tubulin hook method shows that as one moves along the spindle axis from the "west" pole toward the "east," the fraction of MTs oriented with their plus ends distal to the west pole gradually decreases (Fig. 4c). At the spindle equator, about half of the MTs are pointed each way (19). As one moves into the opposite half spindle, the dominant orientation shifts over. The polar orientation of metaphase MTs therefore displays an axis of approximate 2-fold rotational symmetry perpendicular to the pole-to-pole axis and passing through the spindle equator.

Close examination of metaphase MT polarity shows, however, that the orientation of kinetochore MTs is opposite to that previously suspected: MTs that end at a kinetochore have their plus ends at the kinetochore, not distal to it (19,21,25,50,81). This polarity assignment is based on the two structural methods applied to naturally-occurring kinetochore-attached MTs. The initial (and opposite) assignment of kinetochore MT polarity was by inference, and its "confirmation" was by growth rates of heterologous MTs nucleated by isolated kinetochores. We are inclined, therefore, to accept data from structural markers and diagram the polarity of kinetochore MTs with the plus ends at the kinetochores, as shown in Fig. 4d.

Metaphase Distribution of MT Lengths

Spindle structure in overview. Many MTs have one end at the metaphase spindle pole, but only a few have an end at a kinetochore. A systematic search for the other ends of metaphase MTs shows them to be distributed throughout the length and breadth of the spindle. Finding MT ends in the spindle is technically difficult, because they must be identified by electron microscopy. Since fine structure is generally based on thin sections, one can expect that many apparent MT ends are artifacts of microtomy: an MT looks as if it ends, simply because it runs out of the section under study. The accurate identification of true MT ends therefore required serial sections. With this method one can track MTs from one true end to the other through successive slices. A complete reconstruction of the metaphase spindle is available only for the golden brown alga, Ochromonas (86). However, from this and related reconstructions of spindles from Dictyostelium (54), diatoms (53), and a yeast (63), we can describe the metaphase spindle with some confidence.

A computer drawing of the MTs from a very early anaphase spindle of Dictyostelium is shown in Fig. 5a. Each line in the figure is a fourth-order polynomial fit to the points lying along a single MT. Kinetochore-attached MTs are not specially marked, because they could not be identified with certainty in the cross-sections of this spindle. But even so the drawing is too complex to be easily interpreted. Stereo viewing helps, but a more comprehensible projection of the same spindle is shown in Fig. 5b. In this figure each MT is represented by a straight horizontal line; the positions of the MTs' ends along the spindle axis are used to define the horizontal position of the corresponding line segment. The lines are arranged along the vertical axis in order of decreasing length, and then an "envelope" is drawn around the ends of all the line segments. The two envelopes are displayed in Fig. 5b.

Most of these spindle MTs have one end at either one pole or the other. The few "fragments" with both ends free in the spindle are dia-

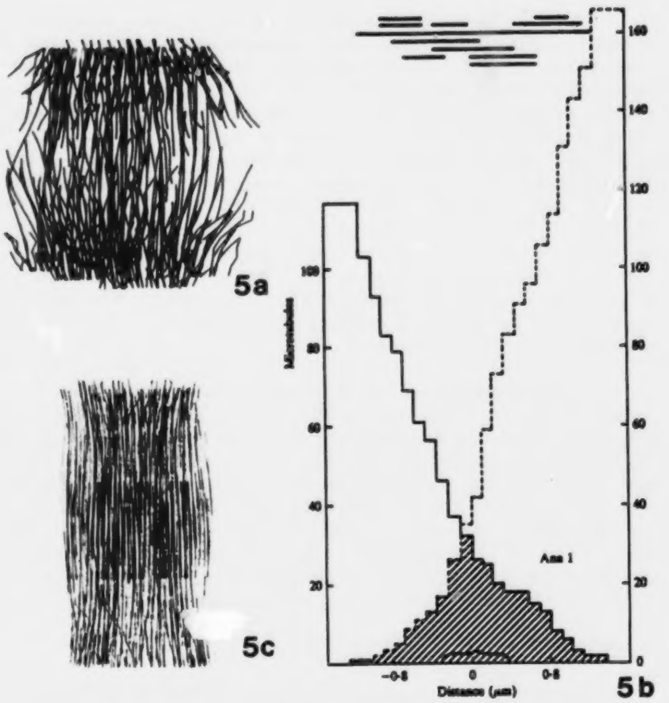

Fig. 5. The arrangement of microtubules in spindles. (a) A computer-generated drawing of the MTs in an early anaphase spindle from Dictyostelium. All MTs are represented as lines or curves projected onto a single paraxial plane. The horizontal dimension has been expanded by a factor of 2 relative to the vertical to spread the MTs out and make them more visible. (b) A different projection of the MTs from the same spindle shown in (a). See text for details. (c) A drawing of the MTs from a metaphase cell of the diatom, Diatoma. Conditions are the same as for (a). (a) and (b) are courtesy of the J. Cell Sci. (c) Courtesy of the J. Cell Biol.

grammed at the top of the figure as separate line segments. The positions of the pole-distal ends of the MTs are heterogeneous; they are scattered essentially uniformly along the length of the spindle. Examination of stereo projections of the spindle in Fig. 5a has shown that the positions of the pole-distal MT ends are also unordered in the 2 dimensions perpendicular to the spindle axis. We conclude that while MT polarity is simple and well-ordered, and while the polar end position of each MT is well-defined, the heterogeneity of MT lengths leads to an essentially random distribution of the pole-distal MT ends.

This early anaphase spindle seems to be constructed from 2 interdigitating arrays of MTs. The longest of the MTs from one pole extends almost to the opposite pole, but the heterogeneous distribution of MT lengths results in a variable extent of MT interdigitation. Some MTs are so short that they are confined to their half spindle of origin. The metaphase spindle from Ochromonas is almost indistinguishable from the early anaphase spindle of Dictyostelium (82). Partial reconstructions of 2 metaphase spindles from Dictyostelium corroborate this image but differ from early anaphase in showing more MTs per spindle cross-section and a larger fraction of the MTs running all the way from one pole to the other (about 10% of the total). This model for the metaphase spindle accounts for the fact, shown in Fig. 4c, that the fraction of metaphase MTs oriented with their plus ends distal to the nearby pole is usually not 100%.

Complete reconstructions of early anaphase spindles from the fungus, Puccinia (83), and the alga, Ochromonas (86), show MT arrangements that are again essentially identical to those of Dictyostelium. While equivalent reconstructions of spindles from higher eukaryotes are not yet available, the data from mammals on MT number as a function of position along the spindle axis are consistent with the model for metaphase structure that emerges from the more complete studies done on small spindles (9,49,52). However, the MT distributions of metaphase spindles in the micronuclei of Tetrahymena differ from those mentioned above in that they lack a dip in MT number at the spindle equator (39). It seems most likely that this difference is a result of the details of the MT length distributions and the fraction of the total MT number represented by those that end on kinetochores.

There appear to be 2 exceptions to our generalizations about metaphase structure. In the spindles of diatoms, the length distributions of the interdigitating polar MTs at metaphase are more sharply specified than those in other cells (53) (Fig. 5c). This degree of order is consistent with the almost paracrystalline arrangement of the pole-associated MTs in these cells at all stages of mitosis (45,84,85,88). The order probably results from the pathway of spindle formation in diatoms. During prophase the spindles of these cells consist entirely of pole-to-pole MTs, but as the spindle moves into the nucleus and begins to interact with the chromosomes, the two half spindles rearrange to make the structure shown in Fig. 5c (88). The sharp distribution of metaphase MT lengths is probably a result of their having run the defined distance from one pole to the other only a short time before metaphase.

The other exception to the general pattern described is found in reconstructions of the spindles from the spermatocytes of crane flies (24). Here MT tracking by serial sections parallel to the spindle axis shows many short MT fragments oriented roughly parallel to the pole-to-pole axis, but scattered randomly throughout the spindle (79). It is not possible to say based on current evidence whether this is a result of a different spindle design or of some preparative difficulty with this material.

Kinetochore MTs at metaphase. Under favorable circumstances it is possible to follow the MTs that have one end on a kinetochore to ask where their other ends lie. In Saccharomyces and Dictyostelium at least one MT that ends on a kinetochore has its other end at the pole (54,63). Serial-section fine structure studies on Ochromonas (87) and the diatom, Hantzschia (85), also show at least one MT running from the kinetochore to the pole, although the bundle of MTs that points toward a kinetochore contains some pole-associated MTs that do not end on the kinetochore and some kinetochore-associated MTs that do not extend all the way to the pole.

This point has been most carefully studied in mammals where the bundle of MTs connecting a kinetochore to its pole has been reconstructed by MT tracking in Chinese hamster ovaries (93) and in rat kangaroo cells (66). In the latter case, the number of spindle MTs was reduced by cold treatment, which solubilizes most of the MTs not in the kinetochore fiber. The cold stable MT bundles that have been reconstructed contain about 30 MTs still attached to the kinetochore; about half of these run all the way to the pole, while the rest end at random. The cold-stable bundle also con-

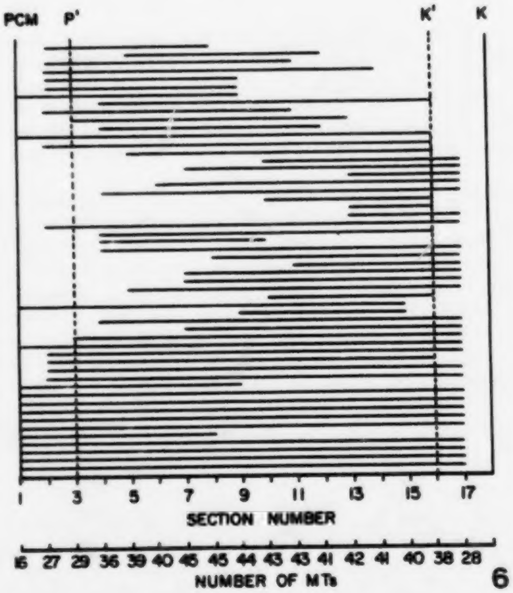

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Fig. 6. . Microtubules in a kinetochore fiber of a metaphase PtK cell. The fiber was cut perpendicular to the spindle axis into sections about 0.25 µm thick, and micrographs were prepared with a high voltage EM. At the left is the pericentriolar material (PCM) extending as far as the line marked P'. The kinetochore extends from K to K'. The axial positions of the beginnings and ends of MTs are represented by the terminations of the horizontal lines. The figure is courtesy of Conley Rieder and Chromosoma.

tains some MTs that have one end in the pole but the other free in the bundle. Still other MTs have both ends free in the bundle (Fig. 6). Kinetochore MT bundles have also been tracked in several cells that lack focused poles: the green alga, *Oedogonium* (74), the micronuclei of *Tetrahymena* (39), and the endosperm of *Haemanthus* (35). In each case the lengths of the kinetochore-associated MTs are heterogeneous. It is not clear in these organisms whether any MTs extend to the pole, because there is no well-defined marker for the pole. Even in these organisms, however, the kinetochore fiber bundle is a mixture of MTs that end on a kinetochore and MTs that do not.

These structural studies suggest that the connection between the chromosome and the pole is complex. Since, however, small spindles contain only 1 or 2 MTs running from kinetochore to pole, and since the polarity of MTs in the kinetochore fiber of all organisms studied is uniform, we are inclined to believe that the complexity of the kinetochore fiber in higher eukaryotes is not important. The simple fiber design revealed by kinetochore MT polarity may convey the more informative view of a chromosome's attachment to the spindle than that seen by detailed structural analysis. Evidence for this opinion was encountered during a near-neighbor analysis of the MTs in the cold stable kinetochore fibers of PtK cells (McIntosh and Rieder, unpubl. observ.). The MTs ending on kinetochores have no special positional relationships with respect to the other classes of MTs in the bundle, so there is no indication that the MTs whose ends lie free and those whose ends are pole- or kinetochore-bound can distinguish each other.

The structural complexity of the mammalian bundle may result either from a physiological breakage of kinetochore MTs that is tolerated by the mitotic mechanism (at least up to a point), or from artifactual breakage that occurs during the preparation for electron microscopy.

Summary

The studies of MT polarity at metaphase allow us to see an overall simplicity in spindle design. The metaphase spindle is composed of 2 interdigitating families of MTs, one that emanates from each pole. The details of spindle geometry show considerable variation from one species to the next. There is variability in the morphology of the poles and kinetochores, in the length distributions of the MTs, in the extent of MT interdigitation, in the number of MTs present, and in the fraction of the MTs that end on kinetochores. These differences emerge, however, as details that are probably not significant for the general patterns of spindle action. The essence of metaphase spindle design can be presented in a simple diagram, such as Fig. 4d.

PATHWAYS OF SPINDLE FORMATION

Introduction

The formation of the spindle may be considered as 2 processes: (a) the initiation and growth of spindle MTs, and (b) the organization of these MTs, together with the poles, the chromosomes, and other spindle components, into the structure we find at metaphase. Temporally the two processes overlap, but they are very likely to be mechanistically distinct. For simplicity we will treat them as separate events.

Initiation and Growth of Spindle MTs

MT formation in vitro. MTs elongate in vitro by the condensation of tubulin onto polymers according to the following reaction:

$$\text{Tubulin} + \text{A Microtubule} \rightleftharpoons \text{A Longer Microtubule.}$$

As diagrammed in Fig. 7a, the reaction can occur at either MT end, but the rate constants at the two ends are different. Tubulin assembly is accompanied by the hydrolysis of a bound molecule of GTP, but the time of GTP hydrolysis is not rigorously coupled to the time of subunit addition (15). Depending on its rate of assembly, an MT probably contains differing amounts of 2 kinds of tubulin: the GDP-tubulin formed by nucleotide hydrolysis (denoted D in Fig. 7a) and a cap of GTP-tubulin comprising subunits that have recently added and have not yet experienced hydrolysis (denoted T in Fig. 7a).

Spontaneous initiation of MT growth in vitro is a complex process involving the coming together of tubulin oligomers to form the tubular geometry. The activation energy for this event is high, and its details are not well-understood. In spindles most, if not all, MT initiation is accomplished by the centrosome, which lowers the activation energy for polymer initiation and will start MT growth either in vivo or in vitro (Fig. 7b) (46,77). Recent studies on the MT-initiating activity of centrosomes in vitro show that the number of polymers which form depends on the tubulin concentration up to a point, but saturates at a number that depends on the centrosome (10,37,56,68) (Fig. 7b and c). Structural studies on the MTs that grow from a centrosome in vitro suggest that the initiator of

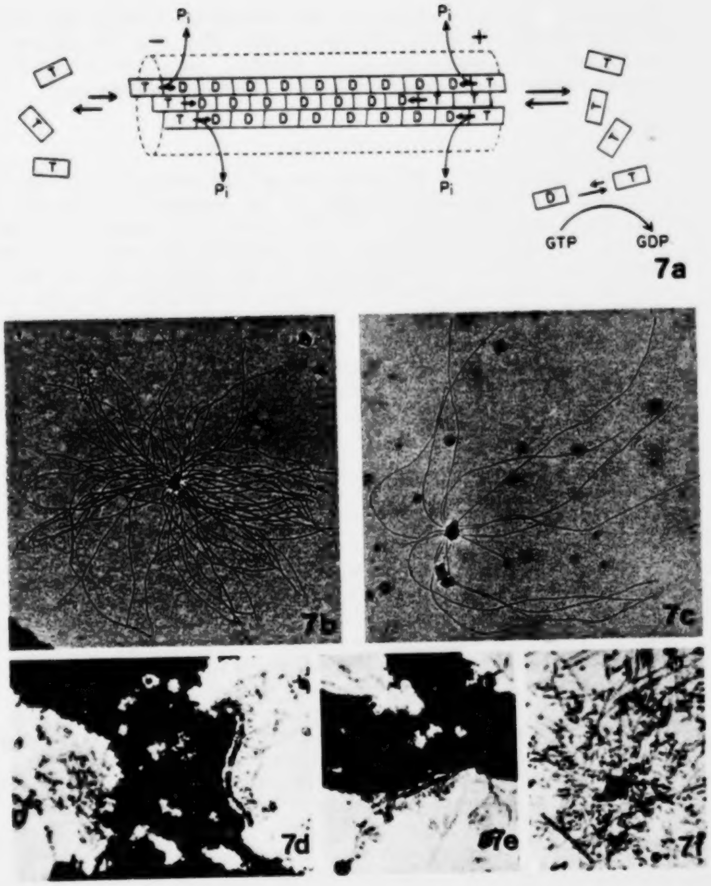


Fig. 7. Microtubule polymerization in vitro. (a) A model for the addition of subunits to either MT end. Subunits labeled T have GTP bound, those with D, bound to GDP. An exchange reaction in solution leads essentially all unpolymerized subunits to have GTP bound. In association with polymerization, GTP is hydrolysed to GDP and Pi, but the reaction does not necessarily occur as the subunit binds to the MT end. Rates for subunit addition and loss are greater at one end (plus end) than at the other (minus end). (b) and (c) MT initiation by isolated centrosomes: they demonstrate that the number of MTs formed depends on concentration of tubulin used (micrographs courtesy of T. Mitchison and Nature). (d) and (e) MT initiation in lysed PtK cells by kinetochores as compared with initiation in the same cell by a centrosome (f). Kinetochore initiation is weaker and less organized (micrographs courtesy of J. Snyder and J. Cell Biol.).

polymerization defines the number of protofilaments per MT (23), so the centrosome probably functions as a set of seeds for MT growth. (For a different view of MT initiation at centrosomes, see De Brabander et al., this Volume.) There is a marked increase in the number of MTs a centrosome will initiate at about the time of nuclear envelope breakdown (38,77; and see

Fig. 4a and b). The mechanism for this 5- to 10-fold change is unknown, but it may result from something released from the nucleus.

The site of subunit addition to MTs growing from a centrosome in vitro is the end distal to the centrosome (41). Since centrosome-initiated MTs elongate at the same rate as MTs growing only at their plus ends (8), and since it is the plus end of these MTs that is centrosome-distal (50), we conclude that the bonds between a centrosome and the MTs it initiates in vitro are strong enough to block insertion of tubulin subunits at the centrosome-proximal ends of the MTs. It is not yet known, however, whether the same condition pertains in vivo.

Kinetochores too can initiate MTs in vitro (8,80,82), although the process is much less efficient than polymer initiation by centrosomes (77). The MTs grown from kinetochores in vitro are not always perpendicular to the plates of the kinetochores (Fig. 7d), as one sees in vivo (Fig. 2), so the initiation reaction in vitro may not be physiological. However, the whole process of kinetochore attachment to MTs in vivo has not yet received the attention it deserves (see below).

Spindle MT formation in vivo. Spindle formation has been studied in living cells by polarization optics. As bundles of MTs form, they give a birefringent retardation to plane-polarized light (depending on their orientation relative to the plane of polarization). This effect can readily be observed and quantified with a good polarizing microscope (reviewed in Ref. 31 and 32). While such an assay for tubulin polymerization is indirect, subsequent work by electron microscopy and immunofluorescence has confirmed most aspects of the picture seen by polarization optics (Fig. 8). In vivo, MTs grow predominantly from the centrosomes. The separation between the centrosomes at the time of spindle formation is variable, and the resulting pathways of morphogenesis differ (49), but the end point of the different paths is always a metaphase spindle. When the poles are distant at the initiation of spindle MT growth (as usually occurs in the zygotes of marine invertebrates and sometimes in cultured somatic cells), then the initial growth of MTs on each centrosome is radially symmetric, forming structures known as asters (see also Fig. 4b). As the centrosome-initiated MTs interact with the chromosomes, they lose their radially symmetric distribution. Except in the spindles of early embryos where the ooplasm includes enough tubulin to form many spindles, the MTs pointing away from the chromosomes shorten while those pointing toward the chromosomes and the opposite pole elongate (Fig. 8b and c).

One cannot yet say for sure whether MTs are nucleated from kinetochores during normal spindle formation. During early prometaphase in spermatocytes of *Drosophila* the kinetochores facing a nearby pole have MTs attached, while the kinetochores facing away are bare (16). Mammalian cells recovering from cold treatment show the same arrangement (67), suggesting that kinetochores get their MTs from the poles. When kinetochores in fertilized sea urchin eggs are kept distant from the centrosome, the centrosome initiates MTs, but the kinetochores do not (76). There is, however, evidence that kinetochores can initiate MTs in vivo under some circumstances. Cells treated with sufficient concentrations of Colcemid (92) or nocodazole (18) will contain no MTs as they enter mitosis. When the drug is washed out, MTs form. Some MTs initiate in the vicinity of the kinetochores, but like the MTs initiated by kinetochores in vitro, these polymers are not initially perpendicular to the plates of the kinetochore (18,92). Eventually, the cells recovering from drug treatment form functional spindles, and the polarity of their kinetochore MTs is normal (22), but it is not yet known whether the MTs that initiated in the vicinity of the kinetochores are the ones that elongated to become the fibers of the kinetochore bundle. Thus, the significance of kinetochore MT initiation

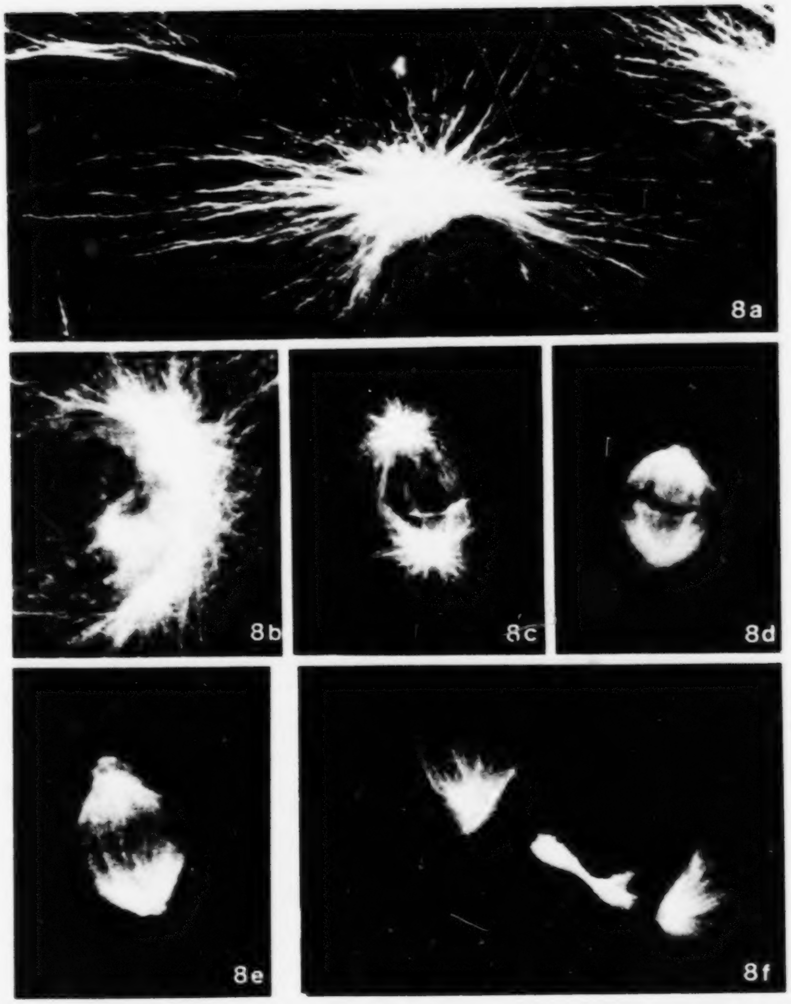


Fig. 8. Microtubule behavior during the cell cycle as visualized by immunofluorescence with antitubulin. Mammalian cells, strain BS/C, are shown in interphase (a), prophase (b), prometaphase (c), metaphase (d), anaphase (e), and late telophase (f). Redistribution of the MTs as a function of time in the cell cycle is obvious. Micrographs courtesy of Jon Izant.

after drug treatment remains to be established. We believe that the balance of evidence currently available suggests that during normal spindle formation the kinetochores capture MTs initiated by the centrosomes, and the MT initiation by kinetochores is not physiologically significant.

MT dynamics in vivo. Tubulin can be labeled nondistinctively with a fluorescent dye (36). This reagent facilitates study of the dynamics of tubulin in vivo (70,73). Fluorescent tubulin microinjected into metaphase cells is rapidly incorporated into the spindle (89). Similar injections into cells at late prophase or early prometaphase show rapid incorporation into aster-like arrays of MTs. In both cases the half-time for incorporation is startlingly fast (about 20 sec). Indeed it is so fast that one tends to doubt the physiological validity of the result. Microinjection can be expected to perturb the cytoplasmic environment; at the very least, the injection of exogenous tubulin will usually change the cytoplasmic concentration of dimeric tubulin, so the rapid incorporation may be an artifactual result of a jump in dimer concentration. For example, one can imagine that simply as a result of an increased concentration of tubulin, most of the spindle MTs will elongate by subunit addition at their plus ends.

This interpretation is put in doubt, however, by a different kind of observation. If fluorescent tubulin is microinjected into prophase cells, and these are returned to the incubator for about a half hour, the preparation contains fluorescent metaphase cells in which the labeled tubulin has had time to equilibrate with the endogenous tubulin pool. The dynamics of tubulin in such cells can then be studied by bleaching small regions of the spindle's fluorescence, using a microbeam of bright blue light from an argon ion laser (70,73). The bleached fluorochrome never recovers, but the bleached spot disappears as tubulin with a bleached fluorochrome exchanges with tubulin bound to unbleached fluorochrome (Fig. 9). The kinetics of fluorescence redistribution after photobleaching (FRAP) can be used to study tubulin turnover at metaphase and during both spindle assembly and spindle function. The half-times for tubulin FRAP are very similar to the

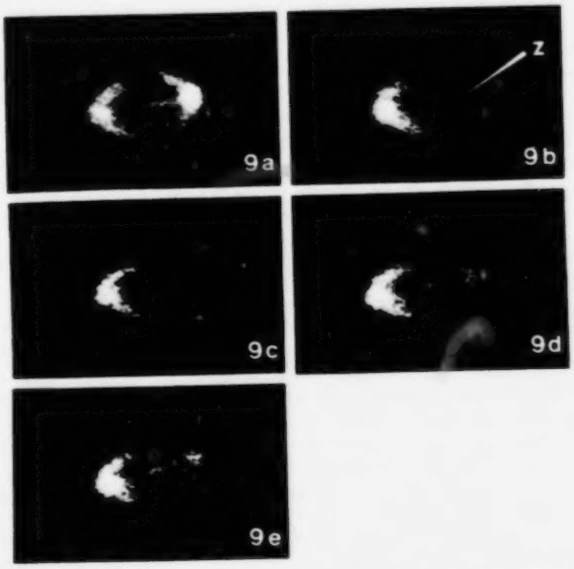


Fig. 9. Microtubule turnover in metaphase visualized with FRAP. (a) A living PtK cell which was injected about 1 hr before metaphase with fluorescent tubulin. The picture is a photograph of a video monitor which is displaying the image obtained from a low light-level TV camera averaged over 16 video frames by a computer. (b) The same cell a fraction of a sec after bleaching of one pole with a laser microbeam. (c-e) Stages in the recovery process. In this particular cell, recovery was not complete, but often it is. Micrographs courtesy of William Saxton.

half-times of fluorescent tubulin incorporation into spindles immediately following injection, suggesting that both experiments are valid measures of the normal rates of spindle tubulin turnover in vivo.

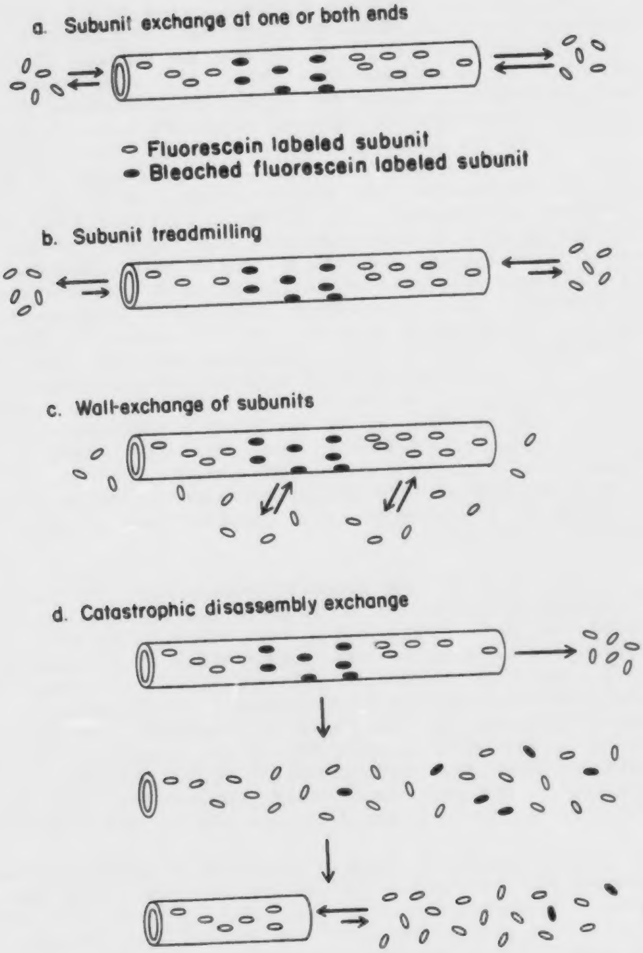
An additional line of evidence implying that spindles are highly dynamic comes from experiments on living metaphase cells viewed in polarization optics. Several different perturbations, e,g, cold treatment, high hydrostatic pressure, or treatments with drugs like colchicine, cause spindles rapidly to disappear (reviewed in Ref. 32). More recently, Salmon and his coworkers have shown that the injection of colchicine will induce such a rapid disassembly of spindle MTs that one is forced to infer the existence of a rapid exchange between subunit and polymer under normal conditions in vivo (71).

Interpretation of MT dynamics in vivo. The structural data presented above place constraints on the ways one can interpret these findings about MT dynamics. Unfortunately, we do not yet have detailed structural information about the sea urchin or mammalian spindles from which the data on tubulin dynamics have been obtained. But the consistency of spindle design over a reasonable breadth of phylogeny gives us the confidence to interpret data on spindle dynamism in the context of the spindle structures seen in the lower eukaryotes.

Tubulin subunit addition and exchange in vitro are always at the MT ends (2,8), so one would expect the same to pertain in vivo. However, the rates of subunit turnover indicated by FRAP in vivo are so fast that something unexpected must be going on. Subunits situated far from an MT end are being replaced with an average half-time of 10-20 sec (70,73). If rapid subunit replacement is occurring by end-exchange, as occurs in vitro (Fig. 10a), then no subunit can be very far from an MT end. If we assume tubulin assembly and disassembly rate constants in vivo equal to those measured in vitro, then the mean MT length in PtK cells must be about 0.1 um to give the measured rate of tubulin FRAP. This is more than an order of magnitude shorter than the mean MT length observed in Dictyostelium, whose metaphase spindle is less than 1/3 the length of a mammalian spindle. Further, direct observation of MT lengths in PtK cells (e.g., Fig. 6) shows these polymers to be several μm in length. We conclude that end-exchange of subunits is an unlikely mechanism for spindle tubulin turnover.

MTs in vitro show a fascinating assembly property at assembly steady-state: they can add subunits at one end and lose them from the other (42). Such a "treadmilling" would greatly increase the rate of exchange of bleached subunits in MTs by unbleached subunits from solution (Fig. 10b). It could account for the observed rate of FRAP, but this model makes a strong prediction about FRAP geometry. Since subunits would be adding at one MT end and coming off at the other, one would expect a flux of subunits down the polymer. Thus, a bleached spot should move either toward or away from the pole as it recovers. Our published evidence does not reveal such a motion (70,73). Salmon and Wadsworth have reinvestigated this question using a narrow slit of bleaching light oriented perpendicular to the spindle axis and located midway between a pole and the metaphase plate (72). The slit-shaped bleach does not move as it recovers, again suggesting that spindle MT treadmilling is unlikely. It is possible that a subset of the MTs, such as those ending on a kinetochore, treadmills at metaphase in vivo, but the data available show that treadmilling is not the explanation for the rapid rate of spindle MT FRAP.

One explanation of the uniform patterns of FRAP would be that spindle MTs can exchange subunits along their lateral surfaces as well as at their ends (Fig. 10c), as suggested by Inoue on the basis of the patterns of loss and gain of spindle birefringence in diverse experiments (reviewed in Ref.


a. Subunit exchange at one or both ends
Fluorescein labeled subunit
Bleached fluorescein labeled subunit
b. Subunit treadmilling
c. Wall-exchange of subunits
d. Catastrophic disassembly exchange

10

Fig. 10. Four models for microtubule turnover. In (a) the subunits exchange randomly at the MT ends, so the replacement of the bleached subunits in the middle of the MT with unbleached ones from solution will be slow. In (b) there is a flux of subunits through the MT as a result of net addition at one end and net loss at the other. This exchange is much more rapid, but predicts movement of the bleached spot which is not observed. Wall exchange (c) would be fast and would account for our observations. Catastrophic disassembly ([d] and as described in the text) is also fast and accounts for the observations.

32). There is no indication from biochemical studies that such a process occurs in vitro, but one can imagine that the methods used to isolate polymerizable tubulin separate the protein from the factors responsible for wall-exchange of subunits. While this model for MT turnover does not seem a priori likely, there is at present no evidence against it.

Recently a completely different view of MT polymerization at steady-state has been introduced by Mitchison and Kirschner (56) and by Hill and his collaborators (30). It is based on the idea of the cap of GTP-tubulin subunits (15) mentioned above (Fig. 7a). Measurements of tubulin assembly and disassembly at different subunit concentrations in vitro are consistent with the idea that the rate at which GTP-tubulin will fall off an MT is rather low, while GDP-tubulin will disassemble 1,000 times faster. When there is sufficient tubulin present in solution to keep on supplementing the GTP-tubulin cap that already exists, an MT is rather stable and will continue to elongate. If, however, the hydrolysis of bound GTP-tubulin catches up with subunit addition, then the GTP-tubulin cap disappears, revealing GDP-tubulin subunits at the MT end. As soon as a given MT loses its GTP-cap, that polymer is likely to disassemble rapidly to zero length (15,30,56). When a centrosome-initiated MT disappears, it probably uncovers an MT-initiating site on the centrosome, so that a new tubule would immediately begin to grow, replacing the one that had just disappeared (Fig. 10d). A centrosome of constant MT-initiating activity placed in a constant pool of tubulin subunits (refreshed by continual conversion of soluble GDP to GTP) should establish a steady-state in which there is a constant pool of soluble subunits with GTP bound, a constant average MT length, and a constant average MT number. Statistical fluctuations in the size of individual GTP-tubulin caps should lead to a constant rate of catastrophic MT disassembly and a constant rate of growth for all MTs still bearing a GTP-tubulin cap.

This view of the assembly steady-state is very different from the treadmilling hypothesis of Margolis and Wilson (Fig. 10b) (42). There is solid, in vitro evidence for both views. The most likely source of the difference seems to be the concentration of MT-associated proteins (MAPs) (91). MAP-rich tubulin in Wilson's hands will treadmill at steady state, while MAP-free tubulin in Mitchison's and Kirschner's hands shows dynamic instability and does not appear to treadmill. The geometry of tubulin FRAP tells us that most spindle MTs do not treadmill, but there is as yet no evidence about the significance of dynamic instability for spindle tubulin turnover. The phenomenon could account for fast FRAP, but data which directly examine this model in vivo are not yet available.

An attractive feature of the dynamic instability model is that it can account for the stability of kinetochore MTs: they would be interpreted as polar MTs whose plus ends have been captured and stabilized by kinetochores. It also accounts for the gradual loss of astral MTs at the expense of spindle tubule growth: astral MTs would lack the stabilizing influences of kinetochore capture or interaction with antiparallel microtubules from the other pole, as described below.

Chromosome Congression and Spindle MT Organization

A description of prometaphase forces. Chromosomes first begin to become organized as they interact with spindle fibers. Often one kinetochore will attach to the spindle before its sister, and in such cases the chromosome is usually drawn toward the pole to which it is attached, suggesting an attractive interaction between a chromosome and a pole. Micromanipulation studies confirm this view, as described elsewhere (Nicklas, the Volume).

Interpretation of prometaphase congression in terms of pole-directed forces. Ostergren was the first to recognize that if the attractive interactions between a chromosome and a pole were of a strength proportional to the distance between the two, then congression to the spindle equator would naturally ensue (61). Experimental evidence for the validation of Östergren's hypothesis has recently been obtained (26). The mechanism for this force, however, is still completely unknown. The force could most easily be explained either by a lateral interaction between the kinetochore microtubules and some other spindle component (a matrix of some kind) or by an elastic component attaching the kinetochore to the pole. The possibility that kinetochore MTs are themselves this elastic component is dubious, because MTs cannot be stretched appreciably without breaking. Unless some factor specific to the spindle can withdraw subunits from along the length of the MT wall, these fibers are most unlikely to behave as plastic or elastic polymers.

Considerable attention has therefore been addressed to the possibility of a matrix component among the spindle MTs (47,65). This could serve either as a medium with which the surfaces of kinetochore MTs could interact to develop a length-dependent force, or as an elastic component that interconnects a chromosome and a pole. In any case, the structural evidence for such a component is sparse. In *Oedogonium* there is an array of slender filaments emanating from the kinetochore (75). These filaments intermingle with the MTs as the kinetochore fibers form. There is, as yet, no information about the composition of these fibers, and equivalent structures have not been seen in other cells. It seems probable that the kinetochore filaments of *Oedogonium* are elaborations of the "corona," the short fibrils found on the pole-facing surface of the kinetochores in many organisms (see Brinkley et al., this Volume). A "collar" of amorphous material surrounding the spindle has been found between chromosomes and poles in the diatom, *Surirella* (84). Here, however, the kinetochores themselves are distended, so it is hard to say where the kinetochores stop and collar material begins. Again, no analogous structure has yet been seen in other types of cells, so the case for a well-defined spindle matrix is weak. The origin of Ostergren's force is still mysterious.

Prometaphase forces on kinetochores are not simply attractions toward the poles. Recent evidence shows that the forces envisioned by Ostergren are not sufficient to account for all aspects of prometaphase chromosome behavior. Monopolar spindles can be made experimentally in some systems (44), and are found by chance in others (5). In both cases, chromosomes attached to a single pole do not draw up close to the pole as Ostergren's hypothesis would suggest. They move to a distance from the pole that approximates the length of a normal metaphase half-spindle, and there they oscillate toward and away from the pole as if they were being both pulled toward and pushed away from the single pole. Similar behavior is seen during normal prometaphase when circumstances favor the attachment of a chromosome to one pole before the other (64). Apparently the spindle can exert one force on a kinetochore that pulls it toward the pole and another that pushes it away. The forces that push are currently no better understood than those that pull.

Metaphase as a balance of forces. At metaphase the chromosomes are comparatively still, although they usually shuttle back and forth near the spindle equator. The net force on each kinetochore is probably toward the pole it faces, because chromosomes on a bipolar spindle that are attached to a single pole move toward that pole. This is true both for spontaneous unidirectional attachments, such as the unpaired X chromosome in grasshopper spermatocytes (59), and for experimental ones, such as those made by laser microirradiation of a single kinetochore (55).

There is abundant evidence that normal chromosome segregation requires a symmetric attachment of each chromosome to the bipolar spindle (discussed by Nicklas, this Volume). Part of this attachment process is the binding of kinetochores to MTs, but an equally significant part is the development of the pole-directed forces. A wealth of evidence suggests that the attachment of a chromosome to the spindle is stable only when under tension

(60). Apparently the force pushing a chromosome away from the pole cannot provide this oppositional force, and it is only when sister kinetochores are attached to opposite poles that accurate segregation is achieved (4). The significance of this property for accurate spindle function is self-evident.

The reaction to the pole-directed forces on the chromosomes must be a chromosome-directed force acting on something else. When metaphase spindles are treated with low concentrations of colchicine (32), with a hydrostatic pressure sufficient to promote slow MT disassembly (69), or with gentle subunit dilution (13), then the poles collapse slowly in on the metaphase plate. Apparently MT depolymerization permits the poles to respond to a chromosome-directed force, suggesting both that the reaction to the pole-directed force on the chromosomes is a chromosome-directed force on the poles, and that the MTs themselves contribute to the forces that hold the poles apart. The facts that some MTs in metaphase and earliest anaphase extend all the way from pole-to-pole and from pole-to-chromosome provide a straight forward explanation for this phenomenon.

It is also likely that MTs from one pole interact with MTs from the other to construct a framework that holds the poles apart. In diatom spindles at metaphase there are no pole-to-pole MTs (53,88), but in the region of MT interdigitation there is a clear tendency for each MT to be surrounded mostly by MTs from the opposite pole (Fig. 11a) (45,87,88). A quantitative analysis of the radial distribution of MTs in these spindle cross-sections confirms that neighbors from opposite poles occur much more frequently than expected by chance, and that these neighbors tend to lie 42 nm apart, center-to-center (Fig. 11b and c). This is morphometric evidence for a specific interaction between antiparallel MTs in the zone of interdigitation. In *Dictyostelium* (54) and *Ochromonas* (87), there are similar indications of interactions between MTs from opposite poles during anaphase when the pole-to-pole MTs disappear.

The interpolar MT bundle probably provides forces to hold the spindle poles apart. Experimental evidence for this role has been obtained from ultraviolet (UV) microbeam studies of spindle stability in metaphase diatoms (40). When one side of the spindle is damaged in the zone of interdigitation, the spindle caves in on that side, and the poles move toward each other (Fig. 11d).

The forces on MTs help to organize the spindle. The tension forces acting on kinetochore MTs will contribute to their alignment with the spindle axis. The compressive forces acting on the MTs that hold the poles apart probably contribute to the bowed shape of the off-axis microtubules in many spindles (e.g., Fig. 5a). Forces within the spindle can therefore be seen as an organizing influence on MTs as well as chromosomes. It is not yet possible to discuss this notion in detail because not enough is known about the relevant forces. Their potential significance for organizing spindle MTs as they assemble is, however, clear.

SPINDLE ACTION DURING ANAPHASE

Introduction

Anaphase chromosome separation usually consists of 2 components: chromosome motion to the poles (anaphase A) and an increase in the separation of the poles (anaphase B). The former includes a disassembly of the kinetochore MTs, while the latter often results, at least in part, from elongation of the MT framework that interconnects the poles. MT assembly and disassembly thus play as essential role in anaphase. It is not obvious,

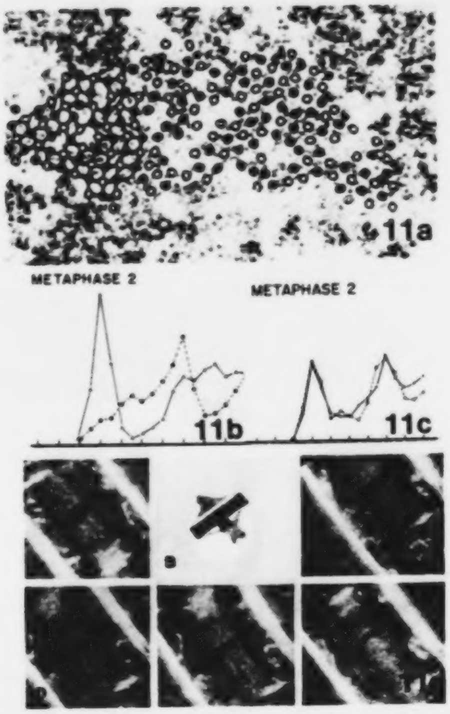


Fig. 11. Interactions between microtubules from the two poles make an interpolar fiber that holds the two poles apart. (a) A cross-section of the midregion of a diatom spindle at metaphase. MTs from the east pole have been colored in black, while MTs from the west pole are unaltered. The tendency of east MTs to surround west and vice versa is indicated by our success in drawing a lattice which interconnects the two MT types at the left side of the figure. A quantitative display of the tendency for alternative packing is shown in (b) and (c). The frequency with which MTs are found at a given distance from one another is plotted as a function of the inter-MT distance, center-to-center. Open circles show east relative to west MTs, and filled circles show each kind relative to itself. Clearly most near-neighbors are of the opposite kind. For (c) the MTs were randomized between the two poles before making the frequency measurement, and while there is still a peak in MT number at 42 nm, center-to-center, it is less marked, indicating that the MTs prefer to have near-neighbors from the opposite pole. (d) A UV microbeam experiment on a diatom metaphase spindle. The dark bar in the first panel is the polarization optical image of central spindle. The spindle is then irradiated, as shown in second panel, and remaining panels show how the spindle buckles when its symmetry is destroyed by micro-irradiation. (a-c) Courtesy of the J. Cell Biol. (d) Courtesy of Roger Leslie and the J. Cell Biol..

however, that they are causal for the motions that occur; they may be simply permissive (51). The following discussion shows that MT movements accompany the polymerization events in ways that make the whole process seem more complex than simply subunit addition and loss.

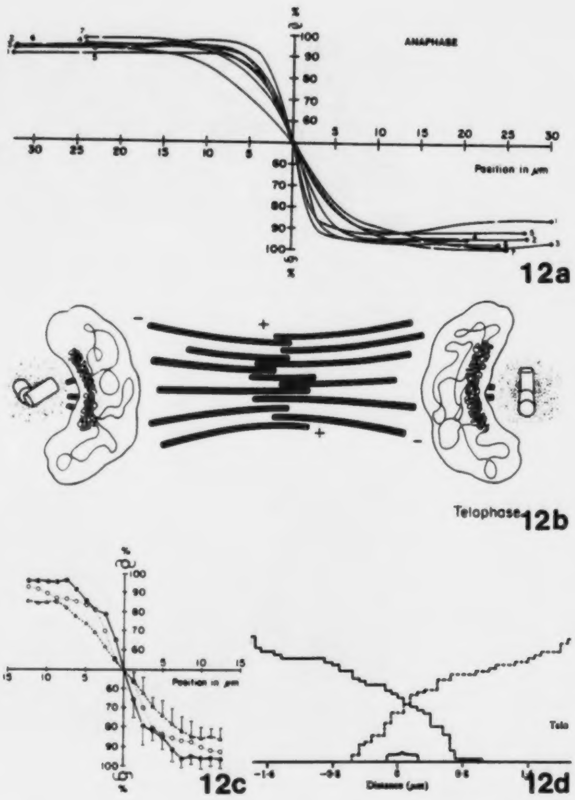


Fig. 12. Spindle structure during anaphase. (a) Distributions of MT polarity in endosperm cells of Haemanthus (courtesy of the J. Cell Biol.). (b) A diagram of the polarity of the spindle MTs in the interzone at late anaphase or telophase. This diagram is applicable to either plant or animal cells. (c) The average polarity distributions at the spindle midplane in Haemanthus for metaphase (triangles), anaphase (open circles), and telophase (filled circles). The vertical marks on the data points are the standard deviations of measurements from at least 7 cells. These data show that the midplane transition from MTs of one polarity to MTs of the other becomes more rapid in late mitosis. Courtesy of the J. Cell Biol. (d) The central portion of an anaphase spindle from Dictyostelium. The reconstruction was accomplished and graphed as for Fig. 5b. A comparison of this graph with Fig. 15b shows that the region of MT interdigitation at the spindle midplane is shorter in telophase than earlier in mitosis. Courtesy of the J. Cell Sci.

MT Polarity, Polymerization, and Movement During Anaphase

MT polarity is conserved during anaphase. Structural assays show that MT polarity is essentially unchanged as the chromosomes approach the poles in anaphase (20,21). The only global polarity change is the disappearance of the small fraction of MTs that run in the direction opposite to the majority in each half-spindle (compare Fig. 4c and 12a) (21). The zone of

MT interdigitation at the spindle midplane stays approximately equidistant between the poles, so the axis of 2-fold rotational symmetry seen during metaphase is also conserved in anaphase (Fig. 12b). There is, however, a subtle rearrangement of the MTs at the spindle midplane. The distribution of MT polarities at this region shifts from a gradual reversal of average polarity to a more abrupt one (Fig. 12c). This observation and the disappearance of the antiparallel MTs from the regions near the poles tell us that each half of the spindle becomes more exclusively unipolar during anaphase. A simple interpretation of this observation is suggested by the changes in MT position that occur during anaphase. Spindle reconstructions based on MT tracking show that the zone of MT interdigitation is now confined to a short region on either side of the spindle midplane (53,54,88) (Fig. 12d).

Anaphase changes in MT polymerization. Electron microscopic analyses of anaphase MT lengths confirm the obvious: kinetochore MTs shorten as the chromosomes move to the poles (54,83). They also tell us something less obvious: that many nonkinetochore microtubules shorten too. The nonkinetochore MT population divides in two at anaphase: one group of polymers shortens while the other elongates (49,54,83,86). The factor that discriminates between these two populations seems to be whether or not the pole-distal end of a given MT is in intimate interdigitation with MTs from the opposite pole. In *Diatoma* the prometaphase-metaphase distribution of MT lengths is bimodal, but during anaphase, the short MTs disappear while the MTs that were long enough to interdigitate at metaphase elongate even further (53). A similar change is seen in *Dictyostelium* (54). We conclude that interdigitation confers stability on MTs and that at anaphase the MTs without this stabilizing influence, including kinetochore MTs, lose subunits.

There are at least 2 possible mechanisms for the stabilizing influence of interdigitation. The regularity of MT spacing in the zone of interdigitation may be the result of specific MT-MT interactions. These would probably stabilize the polymerized state of the tubulin (45). Certainly MT-associated proteins promote MT stability in vitro (58). Alternatively, the stability might be due to a special environment near the spindle midplane. The most likely origin of this environment is the electron-dense matrix seen between the MTs in this region (Fig. 11a). The chemical nature of this matrix is unknown, but the matrix is found in the spindles of most species during anaphase.

In this view of anaphase dynamics, the pole-distal ends of all the spindle MTs (their plus ends) would be potential sites of subunit loss. During anaphase, conditions in the cytoplasm would change to favor disassembly, but those MTs with protected plus ends (the interdigitating MTs) would not respond to the environmental change. As subunits fell from the depolymerizing MTs, the resulting increase in the pool of soluble dimer could drive assembly onto the plus ends located in the special environment near the spindle midplane, causing elongation of the interdigitating MTs.

Some experimental evidence for this view is found in UV microbeam studies on diatoms (40). When the interdigitating MTs are cut by the microbeam just to one side of the zone of interdigitation, the previously stable MTs become labile and depolymerize. The behavior of bits of cytoplasm apparently attached to these MTs suggests that the loss of subunits is from the experimentally-generated plus ends. Meanwhile, the old plus ends that are still interdigitated with MTs from the opposite pole retain their stability; the experimentally-generated minus ends are also stable. Additional evidence for the stabilizing influence of MT interdigitation is found during normal anaphase in diatoms. The interpolar MTs decrease their extent of overlap, and as the pole-distal ends become free, they start to lose subunits, and shorten back toward their pole of origin (78). We conclude that interdigitation promotes MT stability and thus accounts for the apparently bizarre duality in MT assembly behavior during anaphase.

Anaphase changes in position of the MTs in the interpolar framework. The assembly and disassembly events of anaphase result in movements of the ends of some of the spindle MTs, but a detailed analysis of MT position during chromosome segregation reveals additional changes that are not likely to result simply from polymerization events.

Figure 13a, b, and c show early, middle, and late anaphase spindles from *Diatoma*. The structural suggestions that the two interdigitating half-spindles slide apart during anaphase are very strong. It is possible that these images result from a disassembly of tubulin from the pole-distal MT ends and a tubulin assembly onto the pole-proximal ends, yielding the appearance of sliding, but the structural polarity of the MTs and the stabilizing effects of interdigitation make this proposal unlikely.

Two kinds of experimental evidence support the assertion that the interdigitating MTs of diatom spindles slide apart during anaphase. After the interpolar bundle of MTs has been cut with an ultraviolet microbeam in late metaphase, the cell will sometimes enter anaphase. The MT fragments whose plus ends were in the zone of interdigitation are then moved pole-

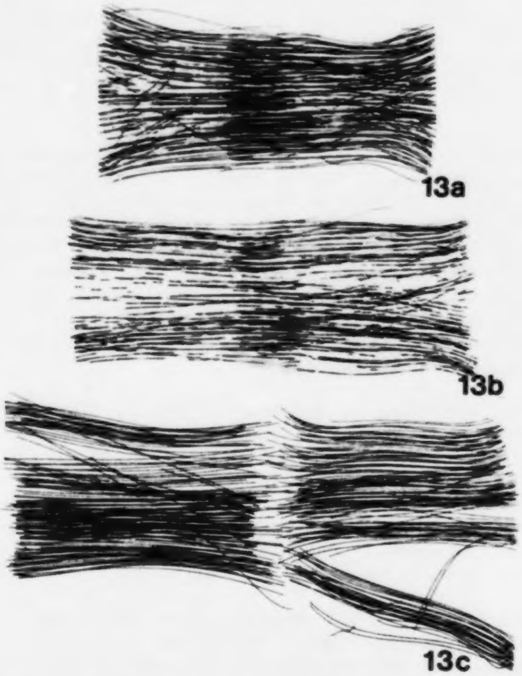


Fig. 13. Structural changes in the central spindle of *Diatoma* during anaphase. These are reconstructions of all the MTs in the spindle prepared as described in Fig. 5. (a), (b), and (c) are early, middle, and late anaphase, respectively. The implication of a sliding motion of the interdigitating MTs is rather clear. Courtesy of the *J. Cell Biol.*

ward, sliding out of the zone of interdigitation (40) (Fig. 14a and b). This observation suggests that the loss of interdigitation during diatom anaphase is probably a result of activities in the zone of overlap itself. Other evidence for MT sliding has recently come from work on isolated diatom spindles. Isolated metaphase spindles will lose their zone of overlap and elongate upon the addition of ATP (Fig. 14c). This motion is poisoned by micromolar concentrations of vanadate ions (14), a property shared by flagellar axonemes where MT sliding is known to result from the action of an MT-associated ATPase called dynein. Taken in sum, these results suggest that in diatoms there is an ATP-mediated sliding of the interdigitating MTs. Under physiological conditions this sliding probably contributes to spindle elongation, but in *Diatoma* the interdigitating MTs elongate as they slide (Fig. 13). There is as yet no evidence about the site of subunit addition to these elongating MTs.

In other organisms the evidence for anaphase sliding of the interdigitating MTs is much less complete. In *Haemanthus* the changes in the MT polarity distribution at the spindle midzone (Fig. 12c) can be interpreted as a result of sliding (19). In mammalian cells both the changes in the distributions of MT number and the shortening of the paraxial length of

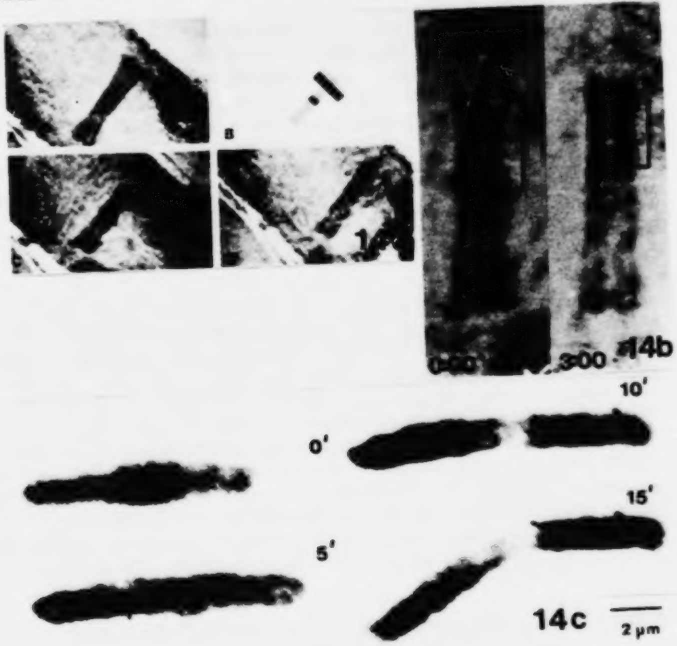


Fig. 14. Experimental evidence for microtubule sliding in the central spindle of diatoms during anaphase. (a) and (b) show a UV microbeam experiment in which the central spindle was cut by the irradiation, yet when the cell entered anaphase, the central spindle elongated some, and the zone of interdigitation at the spindle equator became shorter. Courtesy of Roger Leslie and the *J. Cell Biol.* (c) shows an isolated diatom spindle visualized with polarization optics and a low light-level video camera. Times in min after the addition of ATP are shown in the upper right corner of each frame. These isolated spindles seem to elongate by sliding apart of the two half-spindles. Micrographs courtesy of Zach Cande and *Nature*.

equatorial matrix material between the interdigitating MTs are suggestive of MT sliding (52). Further, anaphase mammalian cells may be lysed under conditions that preserve spindle elongation. This cell model for anaphase B resembles the isolated diatom spindle in that spindle elongation depends on the presence of ATP and is poisoned by vanadate ions (12,14). In *Dictyostelium* there is a small reduction in the extent of MT interdigitation as the interpolar MTs elongate during anaphase (54). In *Ochromonas*, on the other hand, there is no observable change in the extent of MT interdigitation as the poles move apart during anaphase (86). These observations put an experimental question into sharp focus: where do the subunits add to the elongating MTs? If it is at the zone of overlap, then sliding must accompany polymerization to maintain even a constant zone of interdigitation. If it is at the poles, then there is no need for sliding to account for spindle elongation; the partial loss of overlap in some species could be due either to sliding or to a small amount of subunit loss from the pole-distal ends of the interdigitating MTs.

Indirect evidence from the structure of the spindle MTs in *Dictyostelium* suggests that tubulin subunits add to the elongating, interdigitating MTs at their pole-distal ends. Throughout spindle elongation, the MT ends in the zone of interdigitation show an uneven termination of their component protofilaments. In cross-section these ends appear as C-shaped MTs; in longitudinal section, the conventional 2-walled MT image trails off as a single line (54). Such uneven terminations are customarily found at the ends of MTs that are either polymerizing or depolymerizing. All the shortening MTs in *Dictyostelium* show blunt ends. The polar ends of all the MTs are also blunt. Only the pole-distal ends of the elongating MTs are uneven. We infer that the sites of subunit addition to the interdigitating MTs of *Dictyostelium* are their pole-distal ends. If this inference is correct, then there must be extensive MT sliding in the zone of interdigitation to cause the slight reduction in MT overlap that is seen in this organism.

There is some evidence from laser microirradiation at the midregion of a fungal spindle that the later stages of spindle elongation and the nuclear migration that follows mitosis are powered by pulling forces from outside the spindle (1). When the interpolar MTs are cut in late anaphase, the rate of separation of the two reforming nuclei increases, suggesting that these MTs are rate-limiting, not causal, for elongation. We think it likely from the studies described above that the fungal case is one in which postmitotic nuclear migration and anaphase become hard to distinguish. Perhaps an "anaphase C" should be considered to describe those cases of continued nuclear movement in which the forces for motility are no longer from within the spindle, though Heath has proposed that all spindle elongation in fungi is powered from outside the spindle (28).

SUMMARY AND CONCLUSIONS ABOUT THE MITOTIC MECHANISM

Structural and physiological work has defined fairly clearly the mechanisms for spindle elongation. MT growth is important, but at least in diatoms, and probably in other species as well, there is a sliding motor located near the spindle midplane where the two sets of polar MTs interdigitate. The forces for MT sliding are probably generated by interactions between antiparallel MTs in this zone of interdigitation. The rate of MT polymerization is probably important for the rate of spindle elongation because it will define the rate at which track is laid along which the engines can run. In this model, MT polymerization can be thought of as a governor on the rate of spindle elongation, except in those cases, like diatoms, in which sliding runs to essentially zero overlap.

The mechanisms for the movement of the chromosomes to the poles are far less clear. MT disassembly is clearly involved, but intuitively this seems more likely to be a governor than a motor. If we knew where the kinetochore MTs lost subunits, it would help to define the character of the motors for anaphase A. For example, if the noninterdigitating MTs all lose subunits at their pole-distal ends in anaphase, the kinetochore is implicated as a mechanochemical structure that can allow the loss of tubulin while still retaining its hold on the MT end. If subunits are lost at the polar ends, the kinetochore will be merely an MT attachment site and the forces for chromosome movement will be generated elsewhere. We are currently using fluorescent tubulin to determine the sites where spindle MTs gain and lose their subunits.

One of the strongest limitations on our knowledge of spindle mechanism is the paucity of knowledge about spindle protein chemistry. Tubulin is clearly an essential spindle protein, and there is evidence that a 200,000-dalton MT-associated protein is important for spindle stability in mammals (33). For several other proteins known to be present in the spindle, such as calmodulin (90), there is as yet no evidence that they play an essential role in mitosis. A rigorous understanding of mitotic mechanism will certainly require that we achieve a better understanding of mitotic chemistry than is currently available.

ACKNOWLEDGEMENTS

I thank numerous colleagues for their generous permission to use illustrative material, as acknowledged in the figure legends. Our work reviewed here was supported by grants from the American Chemical Society (CD-8), the National Science Foundation (PCM-8014549), and the National Institutes of Health (GM-33787).

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DISCUSSION

FORD: Is it likely that there is a difference in MT-associated proteins (associated with kinetochore MTs as opposed to the others) that would account for this ability to explode? I think that the other evidence you suggested would explain that if you could prove it.

MCINTOSH: There is in fact some good evidence for a difference in the protein composition of kinetochore fiber bundles and other MT bundles in the spindle. The first evidence for this that I know of came from Michael Welsh, Tony Means, and Bill Brinkley working with calmodulin, in which they show that calmodulin during prometaphase--metaphase is associated with the kinetochore fibers. Then as those fibers shorten during anaphase, it stays in the half-spindle between the chromosomes and poles as they withdraw. I do not know of any other protein that behaves that way, but if one does, it is very likely that numerous ones do.

FORD: We first of all see MTs around the nuclear membrane. Then as we go into metaphase we still, to a certain extent, see that the perimeter or the circumference (I do not know exactly which) of the nuclear membrane seems to be retained at the middle of the spindle. Why is the spindle cone-shaped and not collapsing in most systems, though obviously in the Dictyostelium system it is much straighter?

MCINTOSH: There is a lot of variation in spindle morphology when you look at the details of the spindle across phylogeny. It is a fascinating subject. In fact, people have tried to use it to trace phylogenetic interrelationships. My impression is pretty much that any generalization about form you try to make (except for these very simple things like the ones I was trying to emphasize of the bipolarity of the spindle and the emergence of fibers from two centrosomes) can be a problem as you look at diversity, because some spindles are barrel-shaped, some are almost spherical, and some are cylindrical. You know at metaphase there is good evidence for a pole-directed force acting on each kinetochore, and Berns has shown, for example, that if you shoot out one kinetochore, the chromosome will move in the opposite direction at metaphase. So there is tension at metaphase, and that tension has to have an equal and opposite force somewhere. There is pretty good evidence that this force is acting inwards on the poles. That is if you solublize the spindle with low concentrations of colchicine, the spindle will slowly collapse in towards the equator. So I presume that just as there are pole-directed forces on the chromosomes, there are also chromosome-directed forces on the poles. If you ask yourself why don't the poles collapse, well it must be that there is some kind of restraining force, and that is going to tend to make a bowing out of the spindle. I think that when you look at the subtleties like this, that is probably where shape comes from.

FORD: I was wondering whether there may be any known association between the frequency of aneuploidy and the shapes of spindles?

MCINTOSH: Well, the trouble is that the only place where there is this nice mitotic data on frequency of aneuploidy that I know about is yeast. I guess there is something on Drosophila as well.

RESNICK: I have three questions. The first two concern the beautiful description of the distributions of the MTs and their lengths as a function of distance from the centriole or paracentriolar region. If I remember, there was a big difference between, say, the left centriole and the right centriole in terms of the numbers. One was an average of about 150 and the other was around 100. Do you see that frequently and might that account for some aspects of aneuploidy?

MCINTOSH: The spindle is always roughly symmetric, but it expresses its biological nature by never being very crystallinally symmetric. So yes, I think that is a possibility. I am not swept away with it as a possibility because it is a global effect on one side. I would have looked for aneuploidy at things that are more on the symmetry of the chromosomes themselves, rather than on the symmetry of the spindle.

RESNICK: When you have the MTs that stretch all the way across from the centriole to the other side, do they actually attach to the paracentriolar material on the other side, or are they just flopping around there?

MCINTOSH: All I can say from fine structure, of course, is that they end there, which is not very satisfying. I know of no good evidence for attachment. There is some evidence that they support the poles and keep them apart, serving like struts. There is nice structural evidence for dissimilarity between the two ends. That comes from Byer's work on yeast which you are probably familiar with, where if you look at the MTs that end at a pole, some of them have a specialized ending and others do not. One presumes that the specialization is either the minus end, and that is the centrosome from which the two grew, or it is the plus end, and that is the one into which it is impinging. So there is a symmetry, but its functional significance is unclear.

RESNICK: Are the MTs initiated at the kinetochore, and if not, where do they come from? Is it possible that along with the catastrophe theory, maybe there is a breakdown of MTs in the middle, and that somehow these attach to the kinetochore?

MCINTOSH: There is some beautiful work from Mitchison and Kirschner reported at the cell biology meetings, and in press in journals at this point, in which they show that MTs that grow from a centrosome can bump into a kinetochore by chance. They show this by mixing the two in vitro. When they bump, the kinetochore binds. If you then take that mixture, which is a pole with MTs sticking out in all directions, and a kinetochore binding to the plus ends of a few of the MTs, and dilute the system so that the dimer concentration drops way down, all these MTs from the centrosome disassemble. But the one or two that are bound with their distal end of the kinetochore are stable and do not disassemble. So there is nice in vitro experimental evidence that association with a kinetochore stabilizes a MTs to disassembly.

CHARACTERIZATION OF A TIGHTLY CENTROMERE-LINKED GENE ESSENTIAL FOR MEIOSIS IN THE YEAST *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

The centromere region in the yeast *Saccharomyces cerevisiae* is characterized by short DNA fragments, less than 1,000 bp in length, that are capable of stabilizing entire chromosomes throughout mitotic and meiotic cell divisions. The *CEN* fragments are organized in a unique chromatin structure and are surrounded by ordered arrays of nucleosomal subunits. RNA transcripts are found 200-300 bp from the centromere, and lie within this ordered chromatin array. No transcripts have been detected through the centromere itself.

We have examined the expression and cellular function of a tightly centromere-linked transcript on chromosome 11, (*CEN11*)L. The (*CEN11*)L transcript is present at constitutive levels throughout the mitotic and meiotic cell cycles. Disruption of the coding sequences in vivo has no effect on cell viability or mitotic growth, but the cells are unable to sporulate. Genetic complementation with known mutants in sporulation (*spo10*, *spo13*) has defined (*CEN11*)L as a new locus that appears to be required during both meiotic segregation divisions.

INTRODUCTION

The transmission of genetic material to progeny cells during the cell cycle is an essential process that proceeds with a high degree of precision in all eukaryotic organisms. The strategy for executing this process involves the establishment of a spindle apparatus that interacts with specific regions along the eukaryotic chromosome: the centromeres. The assembly of components into the eukaryotic spindle has been studied in a variety of in vitro systems to elucidate the structure and function of this highly coordinate process (11). A complete understanding of how the spindle and chromatin DNA interact necessitates the ability to manipulate the components of the assembly apparatus in vivo. The yeast *Saccharomyces cerevisiae* is particularly suited for studies of chromosome segregation and cell division. The life cycle of the yeast is shown in Fig. 1. Yeast can grow stably mitotically in 2 phases, as a haploid with either an *a* or *α* mating type, or as a diploid. Under certain nutritional conditions diploid cells will undergo meiosis and form 4 haploid products. The short generation time (90 to 180 min) and the ability to dissect and examine the individual products of the meiotic event have provided the opportunity to

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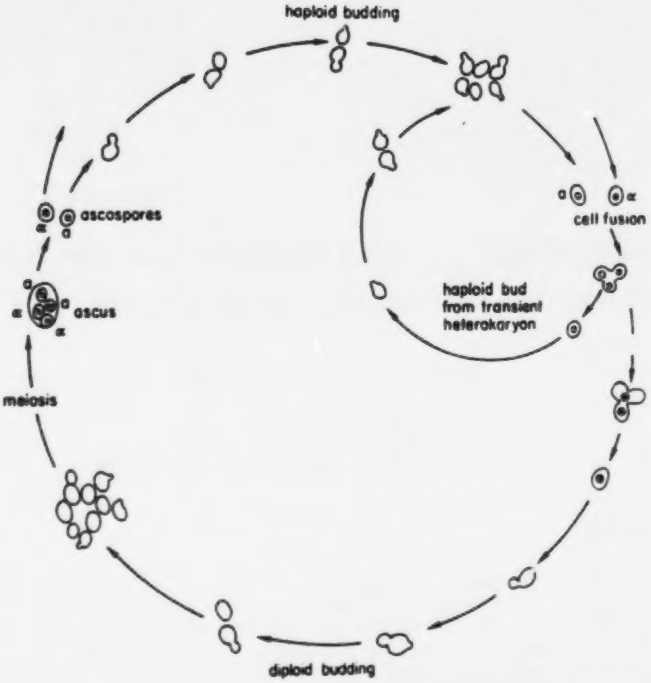


Fig. 1. Life cycle of the yeast Saccharomyces cerevisiae. In heterothallic strains, haploid cells with either an a or α mating type are mitotically stable and will diploidize when confronted with the opposite mating type. The diploid cells are stable. In homothallic strains, haploid cells are unstable, switching mating type to produce diploid cells. Meiosis produces haploid spores.

genetically define a number of cellular processes. More recently, the ability to apply recombinant DNA techniques to this system and manipulate specific genes in vivo allows both direct characterization of gene products biochemically and determination of their cognate function in vivo.

The individual components of the segregation apparatus that have been isolated from yeast include the mitotic spindle, spindle pole bodies (SPB), and the centromeres. The mitotic spindle contains 2 sets of microtubules, one from each SPB that completely interdigitate to form a continuous bundle, and a series of discontinuous microtubules from each SPB (14). Yeast cells from any stage in the cell cycle react with antitubulin antibodies, indicating the persistence of some of these microtubules throughout the cell cycle (12). The isolation of tubulin genes (18) provides the ability to alter the tubulin protein and study its effects on mitosis, as well as defining its role in other cellular processes in yeast. The spindle pole bodies have also been isolated and are authentic microtubule-organizing centers. Isolated SPBs are able to nucleate microtubule assembly in vitro (13), and are analogous to the centrosomes in higher eukaryotes. Control of spindle pole body duplication and separation seems intimately involved in the regulation of mitotic and meiotic cell cycle events. Isolation of SPBs and the subsequent identification of SPB genes will be essential in delineating their role in the control of the cell division cycle.

The attachment site for the distal end of the microtubule in higher eukaryotes is the kinetochore. Kinetochores, as defined by their morphological and spatial relationship to microtubules, are not visible in yeast. However, yeast centromeres, as defined by their genetic segregation properties, clearly represent specific chromosomal sites. The centromeric regions from 5 chromosomes in yeast (CEN) have been isolated (CEN3 [Ref. 5]; CEN4 [Ref. 24]; CEN5 [Ref. 16]; CEN6 [Ref. 19]; and CEN11 [Ref. 9]), and are able to confer properties of chromosome stabilization and proper Mendelian segregation to replicating DNA molecules in yeast (5). Furthermore, when these sequences are deleted from their host chromosome in vivo, an acentric chromosome results and thus, missing a spindle attachment site, is rapidly lost from the population (6). The centromere sequence is therefore required to stabilize the entire yeast chromosome. Chromosome stability is recovered when the centromere DNAs from chromosome 3 or 11 are substituted in either orientation for the CEN3 region in chromosome 3 (3,6). The resulting chromosome segregates normally through mitosis and meiosis in a manner identical to that of the normal chromosome 3. These results indicate that the yeast centromeres are fully functional in either orientation and are not necessarily chromosome specific.

A cytological description of the centromere is more difficult considering the lack of chromosome morphology in yeast. We have examined the molecular structure of yeast centromeres to elucidate the protein-DNA interactions at this unique region of the chromosome fiber. Centromeric DNA is organized in a 220-250 bp chromatin particle that is protected from nuclease digestion in the cell nucleus (1) (Fig. 2). This centromere core particle is structurally distinct from the typical 146-bp DNA-histone core particles that constitute the bulk of chromatin DNA in yeast, as well as in higher eukaryotes. The structure of the centromeric core particle is determined strictly by the centromere DNA sequence, and is associated only with functional centromeric units (1,2,3). Deletion of portions of DNA from the centromere region that inactivate centromere function coordinately disrupts the protected centromere core (2). This region of the chromosome may therefore be the fundamental unit of the yeast centromere. The flanking centromeric regions are characterized by highly ordered arrays of nucleosomal subunits (Fig. 2). These ordered nucleosomal arrays may represent a structurally analogous organization to the highly repetitive satellite sequences characteristic of the centromeric regions in higher eukaryotes.

The direct isolation of DNA sequences from this region of the chromosome allows us to use these sequences as probes for the transcriptional activity of this region of the chromosome. No transcripts have been detected through the centromere itself, however, the regions flanking the centromere along chromosomes 3 (Fig. 2), 4 (24), and 11 (Fig. 2) are all actively transcribed. We have examined the role of the tightly centromere-linked transcript on chromosome 11, (CEN11)L, for an essential gene function by disrupting the coding sequences in vivo and analyzing the phenotype of the mutant yeast cell (25). Haploid cells that cannot make a functional (CEN11)L product (2,200 nt transcript from the centromere region of chromosome 11, Fig. 2) are competent to divide mitotically. Diploid cells incapable of making a functional (CEN11)L product are also competent to divide mitotically, but are unable to sporulate upon exposure to the appropriate nutritional cues. We have been able to order the temporal occurrence of this block with respect to other meiotic landmarks. The cells are indeed committed to meiosis as DNA synthesis and recombination proceed at wild-type levels. Thus the (CEN11)L gene product is required for the first reductional division in meiosis. When (CEN11)L sequences are introduced into the mutant strains on plasmid molecules, the cells are capable of completing the meiotic cell division (25). Thus the gene sequences function in trans and do not have to reside in the host chromosome to provide their

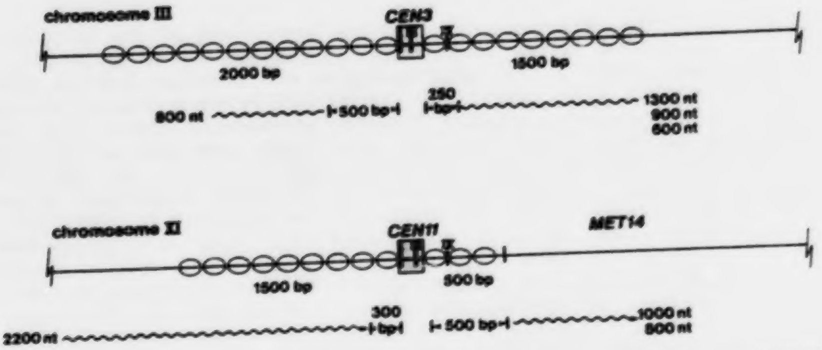


Fig. 2. Schematic map of the structural organization of centromeric chromatin. A map of the chromosomal region surrounding CEN3 and CEN11 is shown in linear form to visualize the central centromere core containing the elements of centromere homology (stippled box, elements I-III), and the ordered array of nucleosomal subunits (ovals) (1,3). The sequence elements I-III are homologous and in identical spatial arrangements between CEN3 and CEN11. A very A+T-rich region lies between elements I and III in both centromeres. The positions of the centromere-linked transcripts are denoted by the wavy lines below the chromosome fiber. Their size is indicated in nucleotides (nt). The (CEN3)R transcripts 1,300, 900, and 600 nt, map within 250 bp of element III, and the (CEN3)L transcript 800 nt, maps within 500 bp of element I. These transcripts lie within the higher ordered nucleosome region flanking CEN3. On chromosome 11 (denoted as chromosome XI in figure), the MET14 RNA complements methionine auxotrophs, and maps to within 500 bp from CEN11. MET14 RNA is initiated outside the higher-ordered nucleosome region and is transcribed in a centromere distal direction (arrowhead). The (CEN11)L RNA is initiated outside the ordered nucleosome region, and transcribed in a centromere proximal direction (arrowhead). (CEN11)L RNA terminates within 300 bp of element I. (Chromosome 3 is denoted by chromosome III in figure).

function. We have analyzed the expression of this transcript during both mitotic and meiotic cell cycles to examine if the transcript is regulated in accord with centromere function during the cell cycle. We show that the (CEN11)L transcript is present throughout both mitotic and meiotic cell division cycles. The proximity of essential gene sequences to the centromere does not interfere with centromere function during the cell cycle.

RESULTS

Cell Cycle Control of the (CEN11)L Transcript

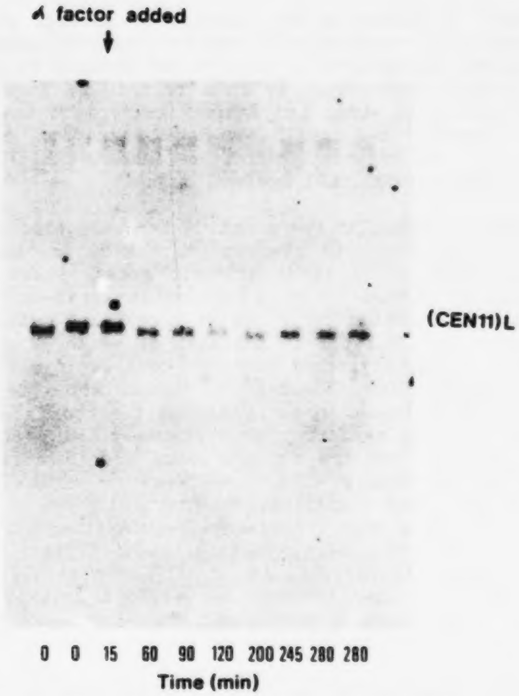
The approach of isolating messenger RNA during specific phases of the cell cycle has been used to identify genes that potentially may be involved in specific processes such as DNA synthesis and sporulation (4,20). Transcriptional activity of the histone genes H2A and H2B, as measured by steady-state levels, has been shown to be tightly linked to the cell cycle (10). Newly synthesized histones are found only associated with the chromatin of S-phase cells, and when DNA synthesis is blocked, histone synthesis is rapidly decreased. Proteins involved in centromere function may similarly be linked to the mitotic or meiotic phases of the cell cycle. Alternatively, transcription may be repressed around the centromere during chromosome segregation in mitosis and meiosis to prevent interference with the spindle apparatus. In this regard, we have demonstrated that transcriptional activation can disrupt centromere function in yeast (Hill and Bloom, unpubl. results). We have therefore measured the steady-state levels of the tightly centromere-linked (*CEN11*)L transcript during both mitotic and meiotic cell cycles.

Haploid yeast of the *a* mating type are blocked at a specific point in the cell cycle G_1, in response to the mating hormone *α*-factor. We have treated yeast cells in logarithmic growth phase with *α*-factor and taken aliquots at various time points after treatment. As the cells progress through the division cycle, cells entering G_1 will be blocked. Cells that were past the G_1 block at the time of *α*-factor treatment continue through the cell cycle until they complete cell division and arrest in G_1. Microscopic observation revealed the accumulation of unbudded cells at this point. Cells begin to recover from *α*-factor arrest at approximately 180 min and proceed through a synchronous round of cell division. Total RNA was isolated from the various time points after *α*-factor treatment as described previously (25), separated by molecular weight on denaturing agarose gels, and transferred to nitrocellulose filters. To visualize specific RNA transcripts, the immobilized RNA was hybridized with ^{32}P-labelled (*CEN11*)L DNA. The autoradiograph shown in Fig. 3 reveals no marked change in the steady-state level of (*CEN11*)L RNA through the mitotic cell cycle. While this does not directly address RNA synthesis at the (*CEN11*)L locus during mitosis, the (*CEN11*)L RNA does not appear to be specifically regulated during the mitotic cell cycle.

The steady-state levels of (*CEN11*)L RNA during meiosis were measured in yeast cells selected for their high degree of meiotic synchrony. The diploid strain MR48 (21) was used in this study. Yeast cells were grown to early exponential phase growth in YPA (1% yeast extract, 2% peptone, and 1% potassium acetate [KAc]) and shifted to a 1% KAc sporulation medium to induce sporulation. Cells previously adapted to respiratory growth in acetate nutrient media result in a more synchronous and more extensive sporulation, approaching 100% in selected strains (23). Premeiotic DNA synthesis occurs in the first 2-4 hr in sporulation medium, with meiotic recombination occurring concomitant with and after DNA synthesis. Four spore tetrads are visible at approximately 8 hr. Samples were taken at 30-min intervals and total RNA was purified as described previously (25), and analyzed as described above. The autoradiograph of (*CEN11*)L RNA during the meiotic cell cycle is shown in Fig. 4. Time points were taken up to 7.5 hr in sporulation medium. When probed with the (*CEN11*)L DNA fragment, no major change in the steady-state level of (*CEN11*)L RNA was seen during meiosis. The larger molecular weight band seen in the autoradiograph is genomic DNA that is used to normalize the amount of nucleic acid loaded in each lane. As during mitosis, there does not appear to be transcriptional regulation of this centromere-linked RNA associated with periods of centromere function.

Ordering (CEN11)L Gene Function In Meiosis

During meiosis, events similar to those in the mitotic cell cycle occur but achieve a different outcome. DNA replication and recombination precede the first meiotic division, where the homologous chromatids separate, but sister chromatids do not. Sister chromatid separation analogous to the mitotic division does not occur until the second meiotic division. Selection schemes have been designed to isolate mutations in genes specific for sporulation (8). These mutant alleles include the *spo*, *spd*, and *mei* genes (7,8,22), and have helped elucidate the coordination of meiotic



.ig. 3. Levels of (CEN11)L RNA in α-factor treated cells. Cells were treated with α-factor as described in the text. RNA was extracted from cells removed from the culture at the times indicated below. The RNA was isolated as described previously, separated by size on a denaturing agarose gel, and transferred to nitrocellulose as described previously (25). Specific transcripts were visualized following hybridization and autoradiography with P^{32}-radiolabelled (CEN11)L DNA.

events (Fig. 5). Diploids entering meiosis mostly have a single, unduplicated SPB. The cells proceed through a round of premeiotic DNA synthesis and recombination, coincident with the duplication of the spindle pole bodies. SPB duplication and premeiotic DNA synthesis are independent events that can be separated by mutational analysis, but their completion is required for the first nuclear division of meiosis 1. The spindle pole bodies separate to form the spindle for meiosis 1, then duplicate for a second time and separate to form two meiotic 2 spindles.

We have determined the temporal order of the (CEN11)L gene function in relation to these previously ordered meiotic events. Yeast cells can be interrupted during meiotic development and returned to mitotic growth on rich medium. With increasing time in sporulation medium, the cells become irreversibly committed to the completion of meiosis and form haploid spores before resuming mitotic growth. In these "return to growth" experiments, (cen11)1 homozygotes were committed to intragenic recombination, but not to spore formation. The mutant cells were blocked at the mononucleate stage (25), and were always able to return to mitotic growth. Thus premeiotic DNA synthesis and recombination do not commit the cells to the meiotic divisions and spore formation. Other mutants that exhibit a similar pheno-

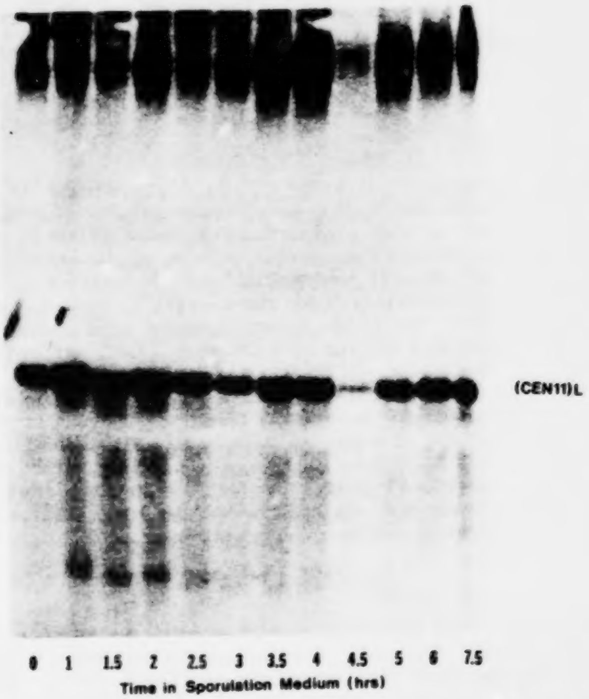


Fig. 4. Levels of (CEN11)L RNA in synchronized meiotic cells. MR48 diploid cells derived from the homothallic SK-1 strain (21) were grown in presporulation medium and subsequently resuspended in sporulation medium (1% KAc) at time 0. RNA was extracted from cells removed from the culture at the times indicated, and separated according to molecular weight on denaturing agarose gels as described in Fig. 3. Radiolabelled (CEN11)L DNA was the hybridization probe. Premeiotic DNA synthesis occurs 2-4 hr after transfer to sporulation medium. Asci first appear after 8 hr in sporulation medium.

type include spo1-1 and spo10-1 (8,17). SPO1 is a tightly centromere-linked gene on chromosome 14, and SPO10 has not been mapped. A (cen11)1 spo10-1 double mutant is able to sporulate at wild-type levels, indicating that these are different genes.

We have examined the requirement for the (CEN11)L gene product at later times in meiosis by utilizing another mutant gene, spo13-1, that bypasses meiosis 1 (8,15). In strains containing this mutation, meiotic recombination is followed by a single meiosis 2-like division. The meiosis 1 reductional division is bypassed, and 2 diploid spores are formed. The (cen11)1 spo13-1 double mutant is unable to complete any meiotic divisions (Fig. 6, right). Wild-type and (cen11)1 spo13-1 double mutants were grown to early logarithmic-phase growth in presporulation media, and subsequently resuspended in sporulation media plus required amino acids. After 4 days incubation at 32°C, cells were first resuspended in 70% ethanol and incubated at room temperature for 30 min. The cells were pelleted, washed in sterile water, resuspended in 0.5 μg/ml diamidino-2-phenylindole dihydrochloride (DAPI) (Boehringer Mannheim), and incubated at room temperature

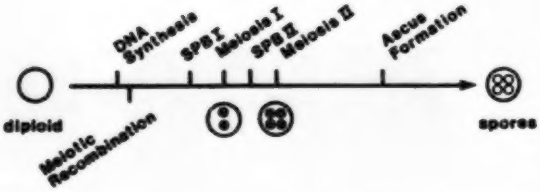


Fig. 5. Schematic diagram of the major events in sporulation. Diploid cells enter the sporulation process during nitrogen deprivation and in the presence of a nonfermentable carbon source such as acetate. The meiotic divisions are preceded by DNA replication and meiotic recombination. Initiation of DNA synthesis occurs approximately 2 hr after shift to sporulation medium, and is complete in 6 hr. The spindle pole body is duplicated for an extended period of time during DNA synthesis. Spindle pole body separation (SPBI) occurs before meiosis 1 (denoted Meiosis I in figure). After meiosis 1, two nuclei are visible with DAPI staining. Meiosis 2 (denoted Meiosis II in figure) is preceded by the second spindle pole body duplication and separation (SPBII). Four nuclei are visible with DAPI staining after the equational division. Asci form around each nucleus and mature spores are visible under the light microscope as early as 8 hr after transfer to sporulation medium, depending on the strain.

for 5 min. The cells were washed once in sterile water, resuspended in sterile water, and visualized using a fluorescent microscope. Normal diploids stained with DAPI are shown in Fig. 6, left. Wild-type cells visualized with fluorescent optics reveal 3 and 4 distinctly staining nuclei that correspond with 3 and 4 spore asci seen in phase optics (data not shown). DAPI-stained, double mutant diploids, seen in Fig. 6, right, reveal only cells in the mononucleate stage. The persistence of the (*cen11*)l mutant phenotype in the presence of the *spo13* bypass indicates that the function specified by (*CEN11*)L is required for sporulation even after meiosis 1.

DISCUSSION

The centromere regions in the chromosomes from the yeast *Saccharomyces cerevisiae* are the first eukaryotic centromeres to be characterized at the DNA sequence level. We have used the centromere and centromere flanking sequences as probes for their molecular structure within the cell nucleus and for the transcriptional activity from this region of the genome. Our findings have revealed that the centromere region in yeast is structurally distinct from the flanking chromosomal arms, however, repetitive satellite sequences that seem to be associated with the centromere in higher eukaryotes are noticeably absent in this region of the yeast genome. Further sequence analysis of the region in yeast may reveal short repeating units (less than 50 bp) that have not yet been detected. A highly repeating array of nucleosomal subunits are characteristic of the flanking centromere region in chromosomes 3 and 11 in yeast (summarized in Fig. 2). These structural subunits lie in a fixed position relative to the DNA sequence that abuts the centromere core, and may resemble the repeating structure associated with the centromeric heterochromatin in higher eukaryotes. The flanking centromere region may therefore be an evolutionary precursor to the structurally differentiated state seen in higher eukaryotes.

This flanking centromere region is also transcriptionally active in

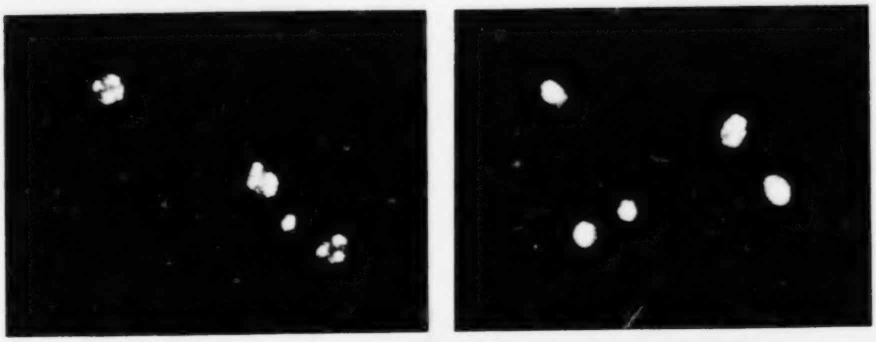


Fig. 6. DAPI staining of sporulated wild-type and (cen11)1 spo13-1 homozygous double mutants. Sporulating wild-type yeast cells were stained with the fluorescent dye, diamidino-2-phenylindole dihydrochloride (DAPI), as described in the text. To the left are wild-type cells after 4 days in sporulation medium viewed with fluorescent optics. Brightly staining dots in the cytoplasm represent mitochondrial DNA. To the right are (cen11)1 spo13-1 homozygous double mutants stained with DAPI after 4 days in sporulation medium viewed with fluorescent optics.

yeast. RNA transcripts that map quite close to the centromere core (within 200-300 bp) have been found in chromosomes 3 (Fig. 2), 4 (24), and 11 (Fig. 2). In no case have any transcripts been detected that are homologous to the centromere core itself. Furthermore, at least one of these transcripts, (CEN11)L, is present constitutively throughout both mitotic and meiotic cell divisions (see Fig. 3 and 4). These results indicate that transcription this near a centromere does not disrupt centromeric stabilization or segregation functions in vivo. When a centromere is placed downstream from a strong promoter in yeast, induction of the promoter can inactivate centromere function as assayed by the ability of these sequences to stabilize autonomously replicating molecules in yeast (Hill and Bloom, unpubl. results). A mechanism to prevent the transcriptional inactivation of the centromere from the endogenous transcripts would seem essential. The (CEN11)L transcript we have examined is transcribed towards the central centromere core, however, other centromere-linked transcripts are transcribed away from the centromere. Strong termination signals that abut the centromere core could prevent transcriptional readthrough and inactivation of the centromere. These termination signals could be specified by the DNA sequence or may result from structural parameters characteristic of the chromatin template.

The ability to direct mutations into a specific region of the host chromosome has allowed us to create a disruption mutation in the (CEN11)L transcriptional unit. The (CEN11)L transcript is phenotypically dispensable in mitosis. In contrast, the (CEN11)L transcript is absolutely required for the meiotic cell division. The (cen11)1 homozygotes are capable of intragenic recombination, but arrest as mononucleate cells. This mutation, along with the previously characterized spo1 and spo10 genes, indicates that the commitment to DNA synthesis and meiotic recombination can be mutationally separated from the commitment to meiotic cell division. From a cytological point of view these results suggest that control of DNA metabolism may be independent from the control of the segregation apparatus, which requires spindle pole body duplication and spindle formation. A cytological analysis of spo1 and spo10 illustrates that the spindle pole bodies remain unduplicated in these cells at the arrest point (8,17). Furthermore, the spol gene is required even after duplication of the spindle pole body in meiosis 1. In this regard, we have also shown that the (CEN11)L gene product is required after meiosis 1. A (cen11)1 spo13-1 double mutant, that bypasses meiosis 1, remains blocked in the mononucleate stage (Fig. 6). Thus whatever the (CEN11)L function may be, it is present at constitutive levels throughout meiosis in wild-type cells and is required for both the reductional and equational segregation divisions.

ACKNOWLEDGEMENT

This work was supported by research grant GM-32238 from the National Institutes of Health to Dr. Bloom.

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DISCUSSION

BOND: Having demonstrated that the centromere sequences are necessary, are they sufficient? The transformation of the chromosomes by putting in the sequences is enough to show that they are necessary, and that is elegant. But when you try to make an artificial chromosome with telomeres and bits of DNA, I would think that the artificial chromosome would be very unstable compared to the wild type. Is there a need for other functions, like the *CEN11* gene product?

BLOOM: Actually there are two issues, and one is that it certainly is true that we do not know that there are not other sequences required for stability. So one thing that we would like to do is to delete a lot of DNA from the centromere region, and then put back a very small piece of DNA and see if that, in fact, is all that is required from that region. The question in terms of the artificial chromosomes is a little more complicated because there may be other reasons that they are unstable, and not just because of the lack of the right elements. For instance, one reason is that a lot of the plasmids that I have been working with are circular plasmids. Well, in fact, we are able to create "real" artificial chromosomes, and these are linear plasmids. These are exactly analogous to linear chromosomes by attaching the specialized structure at the end of the chromosome, the telomere on these plasmids. Short linear chromosomes containing telomeres and centromeres are, in fact, very unstable. We do not think that this is because they do not have the right information, but because there may be a distance requirement between the centromere and the telomere, that in these artificial constructions you are disrupting.

BOND: You don't think then that the CEN11 gene product that you described is one of many examples?

BLOOM: That certainly can also be true.

BARRETT: Do you know whether or not centromere-linked genes are chromosome-specific, or whether or not there is homology between the regions next to the centromeres of different chromosomes? Second, could you speculate as to why you think replication through the centromere region results in loss of function?

BLOOM: We know that the regions flanking the centromere are, in fact, unique DNA sequences. There are no repetitive DNA sequences that we find in the yeast genome. The centromere elements of homology can be thought of as a repeated sequence, but they are 14 and 11 bp. We know that the (CEN11)L gene product can be supplied in trans, so it does not have to be next to the centromere in that location to supply function. We can add it onto a plasmid and recover function. So that says that it is not essential for (CEN11)L to be centromere-linked for its action. Now, why does transcription block? In fact, that is an interesting question and we are trying to look at that. We do not think that it is the transcription per se through the centromere, because other people have done some experiments, namely Peter Phillipson from Basel. He has shown that under a similar transcriptional block, transcripts per se do not go through the centromere itself. We think it is a problem of machinery. The transcriptional machinery is pretty big and the kinetochore is obviously a big structure. So we think that when you try to railroad through this transcriptional machinery, that in some way it is a structural disruption of centromere function.

GAULDEN: What are the dimensions of that region of the centromere that you defined in relationship to the diameter of the single MT that is attached to the yeast chromosome?

BLOOM: Basically we can only speculate on the dimensions because we cannot actually see it, so I cannot measure anything. If we extrapolate from the measured dimensions on the nucleosome core, the nucleosome is 160 bp, and it is about 11 nm (11 x 6 nm). Why? If we extrapolate in terms of the bigger piece of DNA that is protected from nuclease digestion, we can estimate that the centromere is around 20 nm, which is in fact the width of the MT polymer in yeast. So we like to think about that, but we do not know how relevant that is.

THE KINETOCHORE OF MAMMALIAN CHROMOSOMES: STRUCTURE AND FUNCTION IN NORMAL MITOSIS AND ANEUPLOIDY

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ABSTRACT

The kinetochore is a structurally differentiated site on mitotic chromosomes to which spindle microtubules (MTs) are attached. In mammalian cells, the kinetochore is organized into a trilamellar plate and is morphologically distinct from the centromere. Although kinetochores and centromeres are morphologically and biochemically distinct regions, they are functionally linked and necessary for normal chromosome movement and segregation.

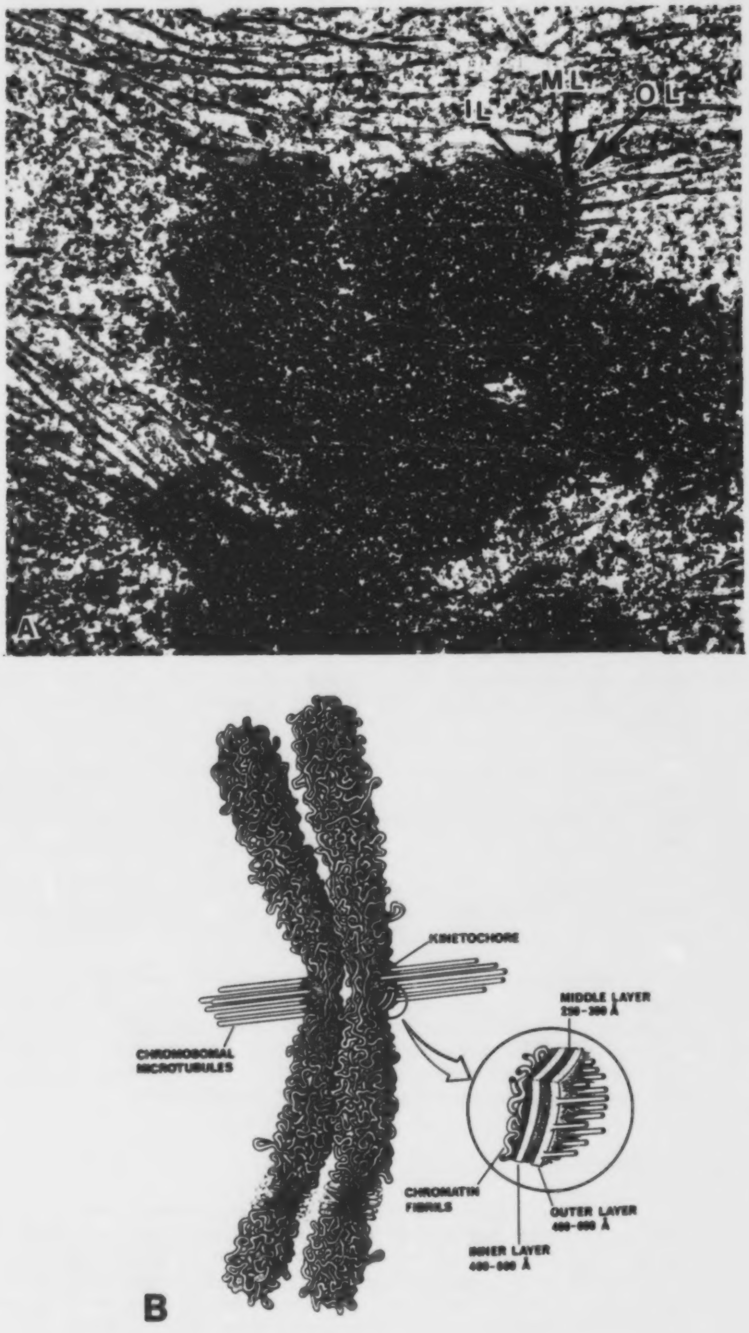
Recent biochemical and immunocytochemical studies suggest that the kinetochore is composed of several polypeptides, DNA, and possibly RNA. The kinetochore plates are composed of tubulin and two antigens of 17 Kd and 80 Kd, as detected by scleroderma CREST antiserum.

Colcemid, a MT inhibitor, also causes reversible rearrangements of kinetochore structure. Mitomycin C binds to heterochromatin and causes the trilamellar plates to become detached from the chromosome.

Diethylstilbestrol (DES), a synthetic estrogen, inhibits mitosis in mammalian cells and causes chromosome lagging or malorientation during recovery. Electron microscopy indicates that DES causes disruption of the mitotic spindle, centriole elongation, and unusual chromosome associations due to interkinetochore microtubules. No apparent damage to kinetochores was noted in lagging or maloriented chromosomes.

INTRODUCTION

The kinetochore functions to attach chromosomes to the mitotic spindle and is required for normal movement and segregation during mitosis and meiosis. The involvement of the kinetochore in nondisjunction and other aberrations of gene segregation in eukaryotic cells has been widely implied, but never clearly defined. Although the frequency of spontaneous error in chromosome segregation is low (less than 10^{-5} in yeast [3]), drugs and toxic agents in the environment may specifically damage the kinetochore producing abnormal chromosome movement and distribution, thereby increasing the frequency of aneuploidy and leading to serious genetic diseases in the human population.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MIDDLE LAYER
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CHROMOSOMAL
MICROTUBULES
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OUTER LAYER
400-600 Å
INNER LAYER
400-600 Å
B

In this report we will review the structure and molecular composition of the centromere/kinetochore region of mammalian chromosomes. In addition, we will evaluate the effects of Colcemid, mitomycin C, and diethylstilbestrol (DES) on kinetochore structure, and determine to what extent kinetochore aberrations relate to abnormal movements and distribution during mitosis (i.e., its role in the mechanism of aneuploidy).

CENTROMERE VERSUS KINETOCHORE: ARE THEY SYNONOMOUS STRUCTURES?

The terms *centromere* and *kinetochore* have been used synonomously in the cell biology and genetics literature for many years. However, in view of our current knowledge of this region and its interaction with spindle microtubules (MTs), it is convenient to view *centromeres* and *kinetochores* as structurally distinct components. Thus the kinetochore is the interface between the spindle fiber and the centromere and is the actual structure to which spindle MTs are attached. The forces which move chromosomes during mitosis and meiosis act through the kinetochore. In addition, the kinetochore may be thought of as one of several microtubule organizing centers (MTOCs) in mammalian cells where kinetochore-associated MTs are organized into polarized bundles (5). The term *centromere*, on the other hand, identifies a broader region of the chromosome. Located at the primary constriction, juxtaposed to the kinetochore, the centromere may be flanked by heterochromatin and composed of DNA which is enriched in repetitive base sequences (20,24). Centromeric chromatin has unique properties which distinguish it from the rest of the chromatin. It is highly condensed and resistant to hypotonic swelling (6,38), and is resistant to nuclease digestion (35,44).

The morphological distinction observed between centromeres and kinetochores should not be construed to imply that the two are functionally uncoupled. On the contrary, recent experiments involving cloned fragments of yeast centromeric DNA showed convincingly that the base sequence of the DNA fragments conveyed mitotic stability and meiotic behavior to replicating plasmids containing these sequences (3,15; Yeh and Bloom, this Volume). Presently, however, the structural and functional relationship between centromeric DNA, the molecular components of the kinetochore, and the interaction of mammalian chromosomes with spindle MTs remains unclear.

ULTRASTRUCTURE, CYTOCHEMISTRY, AND MOLECULAR COMPOSITION

Ultrastructure

The kinetochore of mammalian chromosomes is localized in a small region of the chromosome known as the primary constriction. When this region is observed in ultra-thin section by electron microscopy, the kinetochore can easily be recognized as a plate-like structure located within the clefts of the primary constriction to which numerous MTs are attached (Fig. 1A and B). When examined at higher magnification, the kinetochore plate displays a trilamellar organization consisting of an

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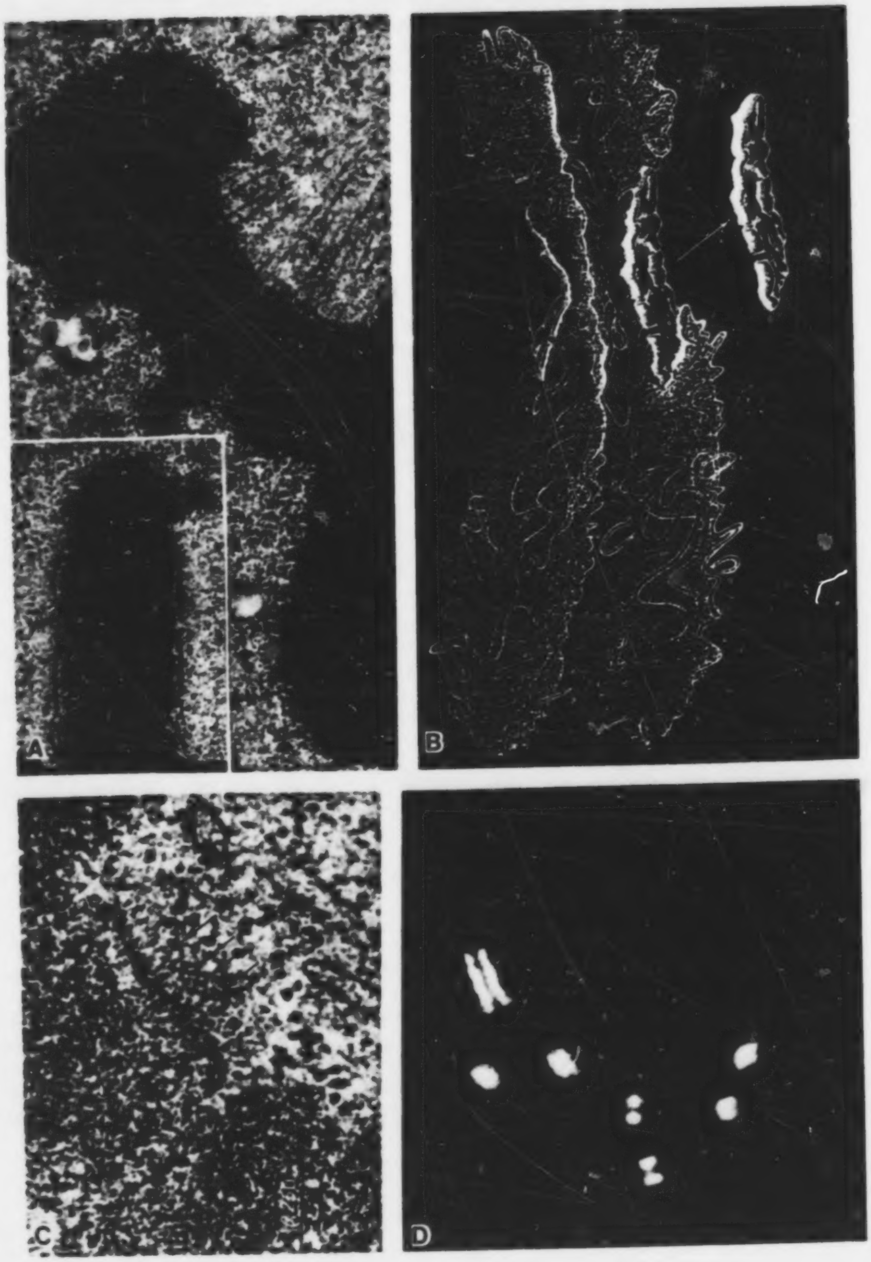
Fig. 1. (A) Kinetochores of metaphase chromosome of rat kangaroo PTK$_1$ cell. IL, inner layer; ML, middle layer; OL, outer layer (from B.R. Brinkley and J. Cartwright, Jr. [1971] *J. Cell Biol.* 50: 416-431). (B) Diagram of metaphase chromosome with kinetochore (modified from S.L. Wolfe [1972] *Biology of the Cell*, Fig. 11-13, Wadsworth Publishing Company, Inc., Belmont, California).


A
B
C
D

inner layer 40-60 nm thick, a lightly staining middle layer of approximately 25-30 nm thickness, and an outer layer also 40-60 nm in thickness (Fig. 1A and B). The inner layer of the kinetochore plate is juxtaposed to the chromatin fibrils and its inner surface is firmly attached to the centromeric chromatin. Thin chromatin-like fibrils extend across the middle layer and appear to cross-link the inner and outer layers. The kinetochore-associated MTs appear to terminate in the outer layer and rarely if ever extend into the middle or inner layers. In addition to MTs, the outer layer contains a fuzzy surface or corona (25) consisting of fine fibrils which appear to form lampbrush-like loops (Fig. 2C).

The overall size and 3-dimensional organization of kinetochores vary with the size of the individual chromosome and the stage of mitosis (see Ref. 37 for review). Kinetochore structure may also be influenced by drugs which disrupt spindle MTs, such as Colcemid and related mitotic poisons (9,10,26,31,39). After Colcemid treatment, the kinetochore plate becomes protracted and attenuated. Examination in the electron microscope indicates that the kinetochore consists of a single-layered plate (Fig. 2A-C) made up of a pair of twisted filaments, 5-10 nm thick (9,10,16,38). Recently Brenner et al. (4) showed by immunoperoxidase staining with anti-kinetochore antiserum that the trilamellar kinetochore was actually present after Colcemid treatment, but that the inner-layer was deeply imbedded into the centromeric chromatin giving the appearance of only a single plate (Fig. 4D). The structural alterations of kinetochores induced by Colcemid and related mitotic inhibitors may have important functional implications since these agents are known to be potent inducers of aneuploidy.

Cytochemistry and Immunofluorescence

Relatively little is known about the chemical composition of the kinetochore, but several cytochemical studies suggest that the kinetochore is composed of DNA, RNA, and proteins. Pepper and Brinkley (33) reported that DNAse I treatment of mammalian chromosomes specifically condensed the plate structure of the kinetochore, while RNAse had little or no effect (Fig. 3A-C). Brinkley and coworkers (6) and Ris and Witt (38) found that the stability of the kinetochore plates was influenced by ionic strength. In the latter study, Ris and Witt (38) found that the outer plate was dissolved in low KCl, and that MTs terminated into a mass of chromatin fibers which stained positively for DNA. The kinetochore can be selectively stained by a procedure which is specific for RNA (36), and the fact that the stain can be abolished by RNAse digestion and cold perchloric extraction (see Ref. 37 for review) suggests that the kinetochore contains RNA. On the other hand, Pepper and Brinkley (33) were unable to detect structural changes or alterations in MT association with kinetochores after digestion with RNAse A or T1 (Fig. 3C).

Through the use of immunocytochemical staining, Pepper and Brinkley (31) found that a polyclonal antibody against 6S tubulin was localized in both plates of the kinetochore. Although the localization of tubulin in

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Fig. 2. (A) Kinetochore from muntjac chromosome after Colcemid treatment. (B) Diagram of metaphase chromosome showing single plate following Colcemid treatment (from B.R. Brinkley, M.M. Valdivia, A. Tousson, and S.L. Brenner [1984] Chromosoma 91:1-11). (C) Kinetochore of Chinese hamster Don cell showing filamentous corona (arrows) (from B.R. Brinkley and E. Stubblefield [1966] Chromosoma 19:28-43). (D) Chromosomes of Indian Muntjac showing kinetochores stained with scleroderma CREST antiserum.

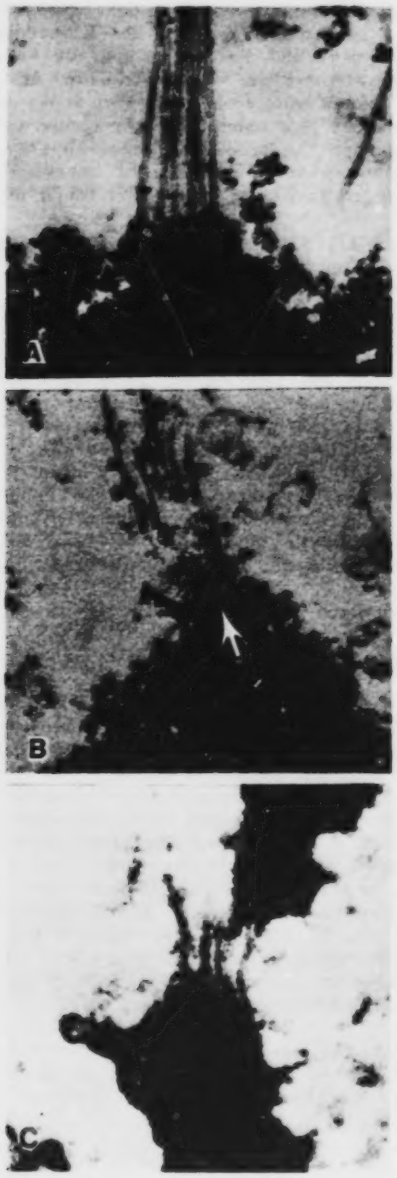

Fig. 3. Kinetochore structure after nuclease digestion. (A) Normal kinetochore, PTK$_1$ cell. (B) After treatment with DNAse showing disorganized kinetochore plate (arrow). (C) Treatment with RNAse T$_1$ had no effect on kinetochore (from D.A. Pepper and B.R. Brinkley [1980] *Cell Motility* 1:1-15).

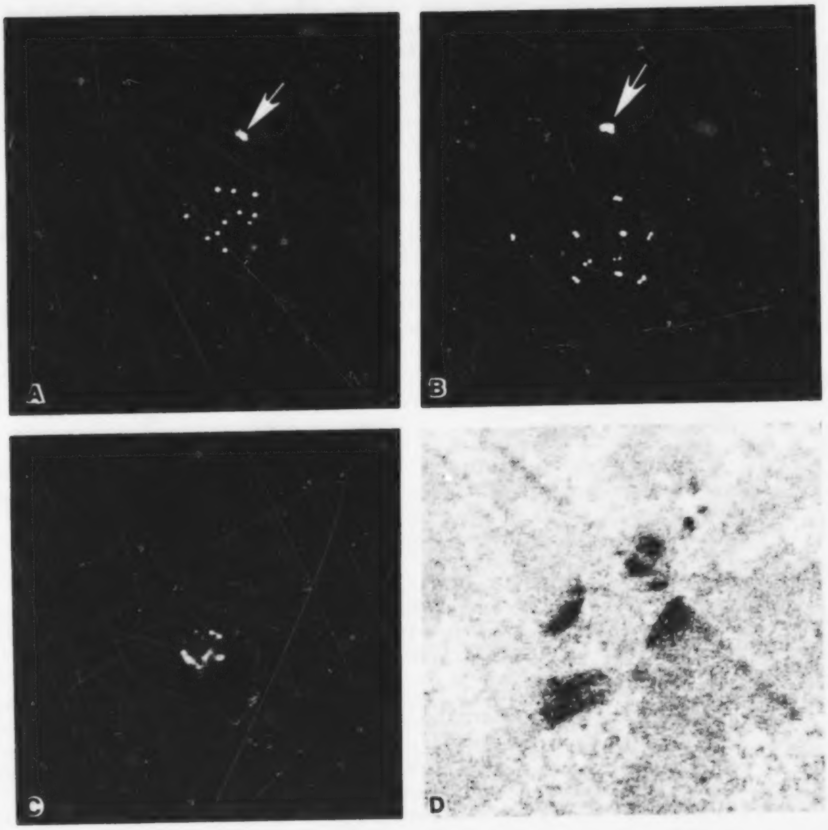

Fig. 4. Kinetochore immunofluorescence with scleroderma CREST antiserum. (A) Interphase showing prekinetochores (arrow points to centrosome stained with second antiserum). (B) Prekinetochores after duplication. (C) Immunoperoxidase-stained kinetochore showing CREST antigens to be localized in kinetochore plates (from Brenner et al. [1981] J. Cell Biol. 91:95-102).

kinetochores might be due to artifactual breakdown of spindle MTs, Pepper and Brinkley (32) found that antitubulin blocked site-specific nucleation of MTs and concluded that tubulin was indeed a structural and functional component of the kinetochore.

A major advance in this area was made possible by the discovery of kinetochore-specific autoantibodies in the serum of patients with the disease, scleroderma CREST (27). Subsequently, Brenner and coworkers (4) demonstrated by EM immunoperoxidase staining that the CREST antigens were localized in both kinetochore plates (Fig. 2D and 4D). The antiserum was also found to stain structures which we termed "prekinetochores" within interphase nuclei (Fig. 4A). The number of prekinetochores corresponded to the diploid chromosome number (4), and by observing the time in cell cycle when the fluorescent prekinetochores became doubled (Fig. 4B), it was possible to determine that kinetochore replication occurred in late S or G_2 phase. Little is known of the mechanism of kinetochore replication, nor has it been determined how faulty replication would influence the distribution of chromosomes during subsequent mitosis. Perhaps the CREST antiserum will serve as a useful probe for further studies in this area.

Biochemistry

Although the kinetochore has been well-characterized at the light and electron microscopic levels, relatively little is known about its molecular composition. This has been due in part to the absence of suitable techniques for obtaining enriched fractions of centromeres from metaphase chromosomes, and to the lack of specific probes for identifying kinetochore-specific molecules. From our limited knowledge based largely on cytochemistry, we assume that the kinetochore is composed of a complex assortment of macromolecules, including DNA, DNA-binding proteins, possibly RNA, and tubulin.

Recently the discovery of human autoantibodies directed against centromeric components (27), and more specifically the kinetochore plates (4; see Fig. 2D and 4), has provided a promising new strategy for biochemical analysis of the kinetochore. Utilizing the discovery of Rattner et al. (35) that the centromere of mammalian chromosomes is highly resistant to nuclease digestion, Valdivia and Brinkley (44) obtained an enriched fraction of the centromere/kinetochore from mammalian metaphase chromosomes. Utilizing a protocol involving extensive digestion of DNA with micrococcal nuclease and dehistonization with heparin, it was found that the residual centromeres stained positively with CREST antiserum indicating the presence of kinetochore plates, a finding supported by EM immunoperoxidase staining. Although the residual chromosomes treated with this protocol contained less than 1% of their total DNA and no detectable histones, they retained scaffold proteins (1) to which was attached the centromere/kinetochore. Next, the preparation was dissolved in detergent (NP40 plus cholate) to release nonhistone proteins, followed by 4 M urea to dissolve the complex. Even after such harsh treatment, some of the residual material retained the capacity to bind to CREST antiserum. A density gradient (metrizamide) was used to obtain a fraction enriched with components which stained with the CREST antiserum at both the light and EM levels. This fraction contained small elecron dense masses or disk-like structures which resembled kinetochore plates of intact metaphase chromosomes. Although it is premature to identify the CREST positive structures as isolated kinetochores, this approach offers considerable promise for further biochemical studies.

When analyzed on 2-dimensional gels, 2 major groups of polypeptides, having molecular weights of 34-40 Kd and 50-55 Kd, were enriched in this purified fraction when compared with bulk chromosome proteins. Very similar distributions of proteins were found in HeLa and CHO chromosomes.

Tab. 1. Kinetochore antigens detected by immunoblotting with scleroderma CREST antiserum.

Polypeptides (Kd)	Species	References
14, 20, 23, 34	Human (HeLa)	Cox et al. (17)
77, 114	Human (HeLa)	Earnshaw et al. (19)
15, 33	Trout (testis) Rabbit (thymus)	Ayer et al. (1a)
19.5	Human (HeLa)	Guldner et al. (21)
18, 80	Human (HeLa)	Valdivia et al. (44)

Using Western transfer techniques (43) Valdivia and Brinkley (45) have identified 2 proteins in the kinetochore-enriched fractions which cross-reacted with CREST antiserum, one with a molecular weight of 18 Kd and another of 80 Kd. Although it is presumed that both peptides are present in the centromere/kinetochore region, the 18-Kd protein is released into the supernatant after treatment with nuclease and heparin. The 80-Kd antigen, however, is tightly bound to a component which was resistant to all treatments and is therefore probably a component of the plate. It will be important to further characterize these kinetochore antigens and determine their arrangement in the centromere/kinetochore organization.

As shown in Tab. 1, other laboratories have also identified antigens which may be components of the kinetochore. Cox et al. (17) utilized a scleroderma CREST antiserum to identify polypeptides of 14, 20, and 34 Kd by immunoblotting, and concluded that these were involved in binding MTs to kinetochores. Earnshaw et al. (19) found that the sera from 9 CREST patients reacted with a 17 Kd and 114 Kd peptide. In a recent report Guldner et al. (21) reported that 18 different CREST sera reacted with a 19 Kd, nonhistone chromosomal protein form HeLa cells. The variations in polypeptides which cross-react with CREST serum are not unexpected in view of the fact that they come from different patients with different titers. Nevertheless, the autoantibodies form scleroderma patients provide useful probes for dissecting the molecular composition of kinetochores and offer considerable promise for identifying important proteins in this component.

EFFECTS OF DRUGS ON KINETOCHORE STRUCTURE AND CHROMOSOME DISTRIBUTION

Colchicine and Related Mitotic Inhibitors

As described previously, colchicine and colchicine-like drugs not only bind to tubulin and prevent MT assembly, but they also significantly alter the structure of the kinetochore at a wide dose range of .06-1.0 ug/ml. The mechanism of this interaction is unknown but may be related to the finding in our laboratory of tubulin within the structure of the kinetochore plates (3). The relationship between colchicine-induced kinetochore damage and aneuploidy induced by colchicine and related drugs deserves further study.

Mitomycin C

The antibiotic, mitomycin C (MC), is known to bind to heterchromatin and to inflict damage to the kinetochore (29). When rat kangaroo cells (strain PtK1) were treated with 1.0 ug/ml for 24 hr, Brinkley and Shaw (8) observed metaphase chromosomes with striking damage to the kinetochore (see also Ref. 7). As shown in Fig. 5, MC treatment resulted in the displacement of both the inner and outer kinetochore plates from the adjacent cen-

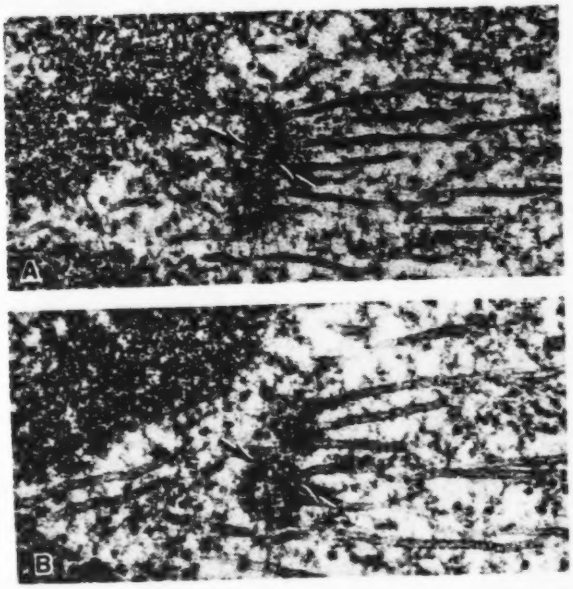

Fig. 5. Kinetochore plates (arrows) dislocated from chromosome (CH) after treatment with mitomycin C (from B.R. Brinkley and M.W. Shaw [1970] Genetics Concepts Neoplasia, Williams and Wilkins, Baltimore).

tromere. These findings indicate that some drugs do selectively alter kinetochore structure and such aberrations could indeed result in the loss of spindle MT attachment and chromosome nondisjunction.

Diethylstilbestrol (DES)

The synthetic estrogen, diethylstilbestrol (DES), has been identified as a carcinogen by Herbst et al. (22) who found genital tract tumors in young women whose mothers had been given DES during pregnancy. These studies were confirmed clinically by Noller and Kurland (28), although the mechanism of how DES induces carcinogenesis remains unknown. DES is also an inhibitor of mitosis (23,40,41), has been shown to induce abnormal anaphase and telophase configuration (18), and aneuploidy (2,12,13, 14,30,34).

In collaboration with T.C. Hsu and coworkers, we have utilized electron microscopy and indirect immunofluorescence with antitubulin and CREST antikinetochore antibodies to investigate the effects of DES on 3 cell lines; Chinese hamster Don, Chinese hamster ovary (CHO), and Swiss mouse 3T3 cells. The aim of our studies was to determine the effect of DES on the ultrastructure of mitotic cells and to compare these effects with Colcemid and related microtubule inhibitors. Moreover, using electron microscopy and immunofluorescence, we wished to determine whether or not DES-induced lagging or maloriented chromosomes contained normal kinetochores and spindle MT association.

In agreement with the study of Satya-Prakash and coworkers (40) we found DES to reversibly arrest mitosis at a relatively high dose over a narrow concentration range (Fig. 6). Examination of DES-blocked cells by electron microscopy indicated some striking differences between these cells and cells arrested with Colcemid (see Ref. 11). Overall, DES blocked bipolar spindle assembly and produced cells with a monopolar organization. However, instead of chromosomes being radially distributed around the single mitotic pole as with Colcemid, most DES-blocked cells contained chromosomes which were grouped together in small clusters of 3 to 5 chromosomes, and appeared to have little association with the pole (Fig. 7). The

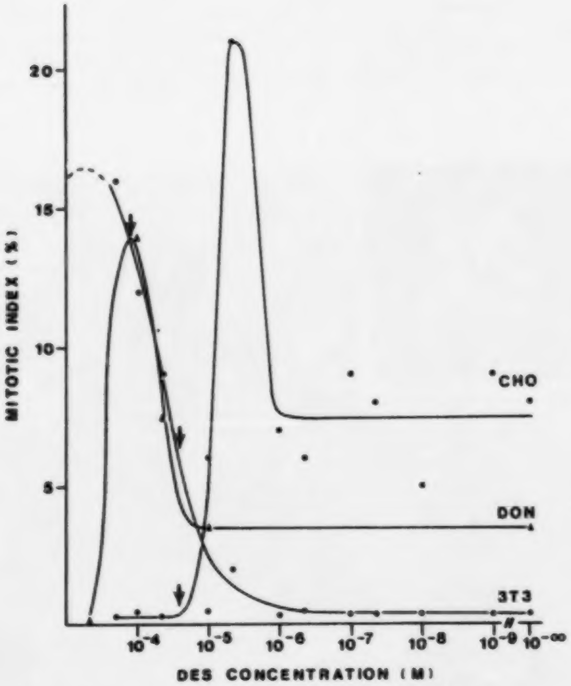

Fig. 6. Profile of mitotic arrest induced by DES at various concentrations. Arrows indicate points below which the mitotic arrest induced by DES is reversible. The mitotic index of 3T3 was not monitored above 2 X 10^{-4} M DES. Dashes represent the hypothetical peak of mitotic arrest for 3T3. Mitotic index was calculated as a percentage of mitotic cells out of at least 200 total cells counted for each drug dosage. Data were graphed semi-logarithmically.

clustered chromosomes were held in association by short MTs which extended from one kinetochore to another (Fig. 8B-D). These unusual kinetochore-to-kinetochore MTs were independent of those associated with the centriole-spindle pole. When DES-blocked cells were prepared for indirect immunofluorescence using tubulin antibody, several aster-like bodies could be seen in the cytoplasm (Fig. 7A and C) representing small clusters of chromosomes held together by interkinetochore MTs.

The kinetochores of DES-treated chromosomes were structurally similar to those of Colcemid-treated cells (Fig. 8B). They consisted of a single plate with dimensions essentially identical to those of Colcemid-treated cells.

DES had an unusual effect on the structure of centrioles in arrested mitotic cells. Many, but not all, centrioles were greatly elongated (Fig. 7B,8A). One member of a centriole pair usually displayed normal morphology with an overall length of 280-300 nm. The other member, presumably the daughter, was greatly elongated with a length of 600-650 nm. The distal end of the elongated centriole was capped with an electron-dense, amorphous material. To our knowledge such elongated centrioles have not been observed in either control cells or cells treated with other mitotic inhibitors. Although Colcemid was found to increase the frequency of basal body

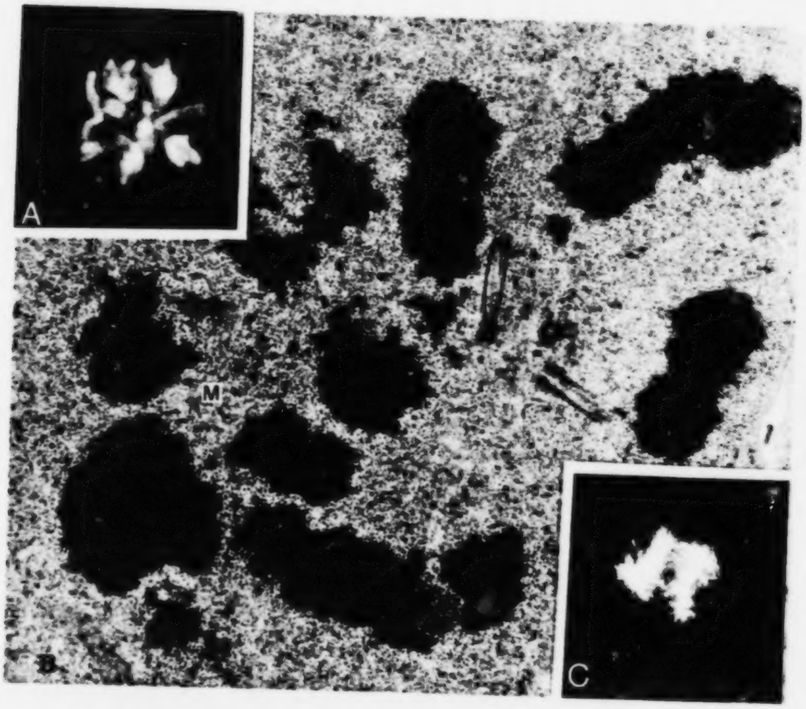

Fig. 7. Chinese hamster Don cells blocked with DES. Note chromosome aggregations and aster-like microtubules representing kinetochore-to-kinetochore microtubules. Centrioles are unusually elongated. (A) Hoescht stain. (B) EM. (C) Antitubulins immunofluorescence showing asters.

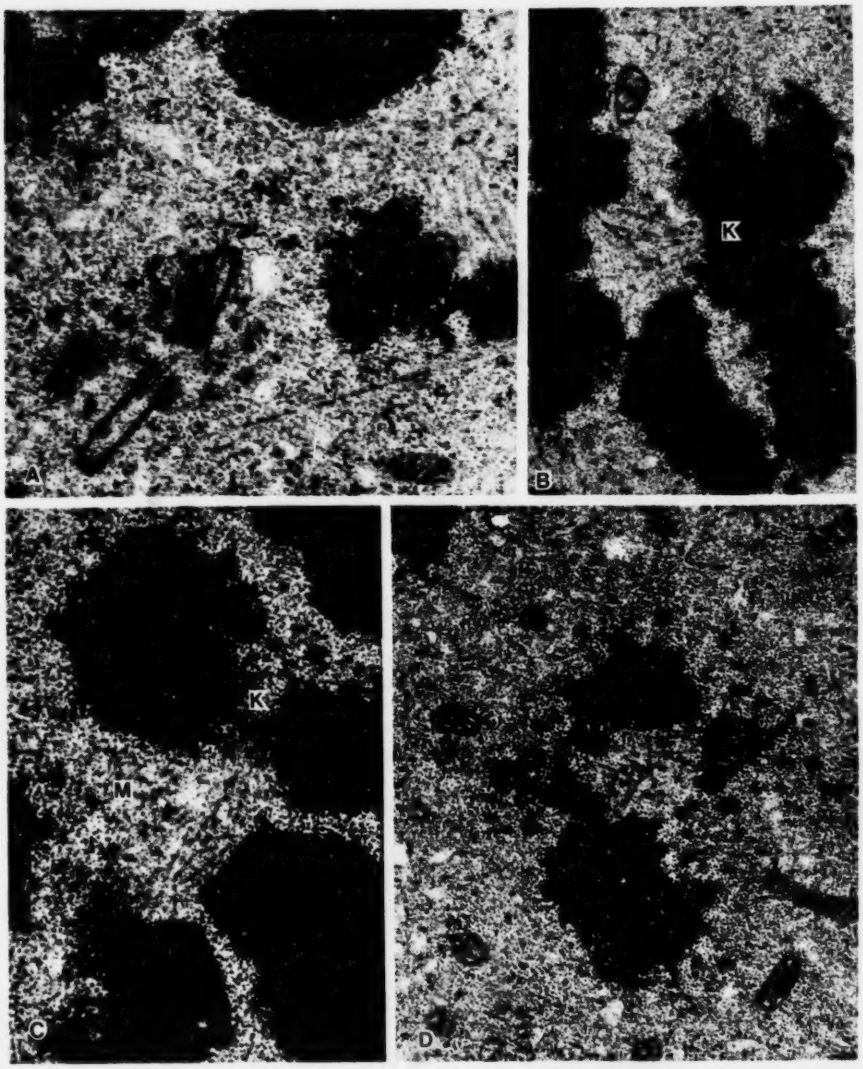

Fig. 8. Electron micrograms of DES-blocked Chinese hamster cells. (A) Elongated centrioles and a normal centriole (CT). (B-D). Chromosome aggregations with interkinetocore microtubules (M) and kinetochore (K).

formation and ciliogenesis in Chinese hamster cells (42), the length and organization of the centrioles during Colcemid-induced ciliogenesis was considerably different from those seen in DES-treated cells, and cilia always formed in interphase cells, not arrested mitotic cells. At this time we have no explanation for these unusual centrioles in DES-treated cells. Further studies are underway and our preliminary observations are mentioned here because they may relate in some way to chromosome associations in DES-blocked cells or faulty chromosome orientation in anaphase during recovery.

When DES was removed from the media and the arrested cells were allowed to recover and resume mitosis, most of the cells formed a bipolar spindle and resumed mitosis within 15-30 min. As shown in Table 2, the frequency of anaphase cells with lagging chromosomes was significantly increased in all DES-treated cells except CHO cells. Also, there was a direct correlation between the number of lagging chromosomes at anaphase and the frequency of multinucleated cells in telophase. Apparently many of the lagging chromosomes reached the poles of the mitotic cells but were not included in the main daughter nucleus, forming micronuclei instead. The selectivity of DES for aberrations in chromosome distribution over other types of damage is indicated by the fact that the frequency of cells with chromosome bridges was the same as in the controls.

The availability of large numbers of cells with lagging or maloriented anaphase chromosomes afforded us an opportunity to examine their kinetochores for possible structural damage, deletion, or loss of kinetochore-associated MTs. Through careful examination of serial thin-sections by electron microscopy followed by 3-dimensional reconstruction of anaphase spindles, it was possible to determine that lagging chromosomes contained bundles of MTs still attached to their kinetochores (Fig. 9A-C).

Tab. 2. Effect of diethylstilbestrol (DES) on segregation of chromosomes and nuclei. Values with identical letter superscripts are statistically equivalent; those with nonidentical superscripts are statistically different. The frequency of anaphase cells with laggards was determined in each case by inspecting anywhere from 150 to 700 cells (unless otherwise indicated); for multinucleate cells, from 200 to 2,500 cells were inspected. Asterisk indicates limited sample. All statistics were computed at the 5% level of significance.

Cell type	DES dosage	Frequency of anaphase cells with lagging chromosomes (%)	Frequency of lagging chromosomes with kinetochores (%)	Frequency of multinucleate cells (%)	Frequency of chromosome bridges (%)
3T3	2.5×10^{-5} M for 24 hr; 45 min reverse	26^{a}	98	21^{a}	6
	Control	8^{b}	100*	6^{b}	8
DON	5×10^{-5} M for 4.5 hr; 30 min reverse	20^{c}	92	11^{c}	5
	Control	5^{d}	--	4^{d}	5
CHO	5×10^{-6} M for 14 hr; 40 min reverse	10^{e}	55	4^{e}	16
	Control	5^{e}	--	7^{e}	14

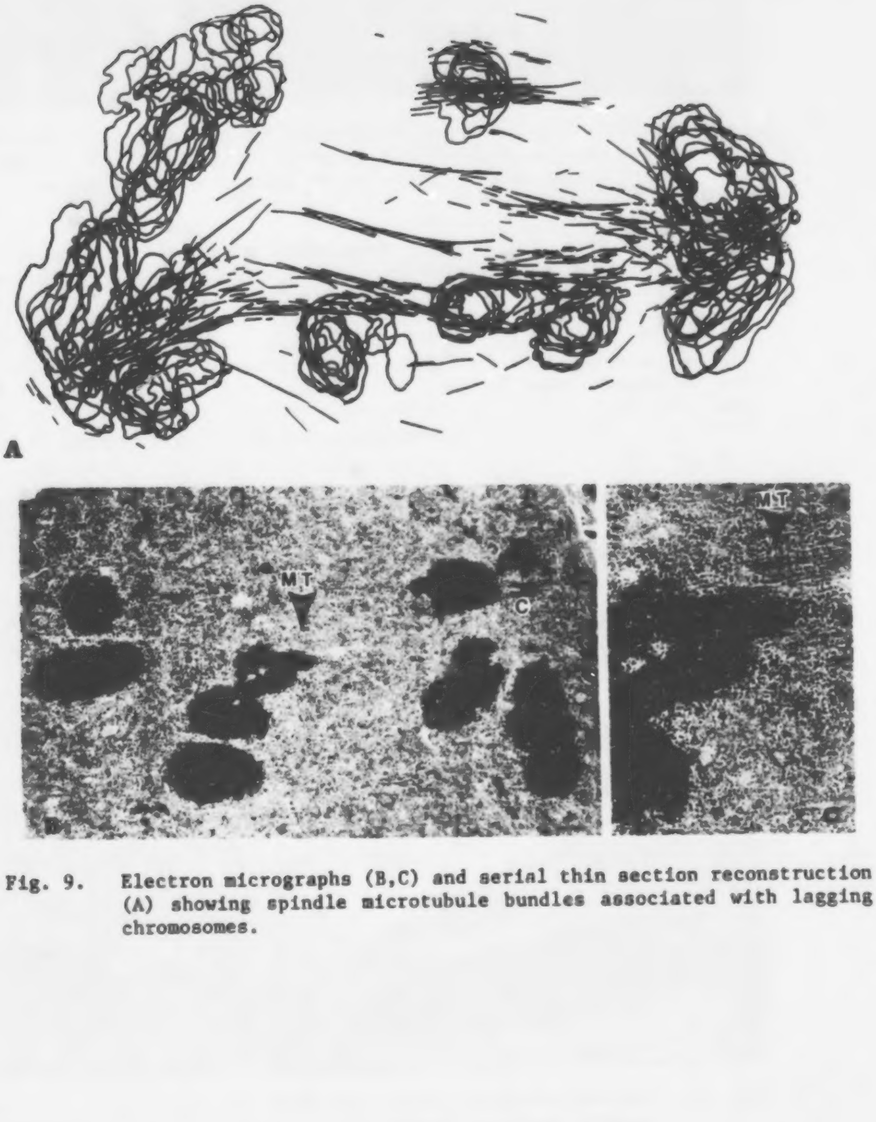

Fig. 9. Electron micrographs (B,C) and serial thin section reconstruction (A) showing spindle microtubule bundles associated with lagging chromosomes.

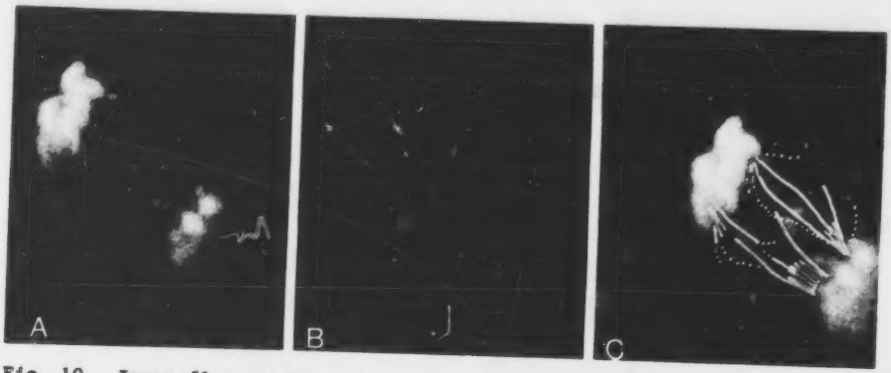

Fig. 10. Immunofluorescence micrographs of the same cell showing spindle fibers associated with lagging chromosome. (A) chromosomes; (B) spindle; and (C) over-lay of spindle on chromosomes.

In addition, the lagging chromosomes were often surrounded by bundles of interzonal MTs (Fig. 10A-C). In most cases, lagging chromosomes appeared to be late in undergoing chromatid separation, but were usually oriented toward the major spindle poles. Our observations indicate that failure of chromosomes to move to the poles in concert with the other chromosomes is not due to the absence of kinetochores or kinetochore MTs. During telophase, some lagging chromosomes were often so late in separating that they were caught in the cleavage furrow, but these too contained bundles of kinetochore MTs intermingled with MTs of the midbody (Fig. 11).

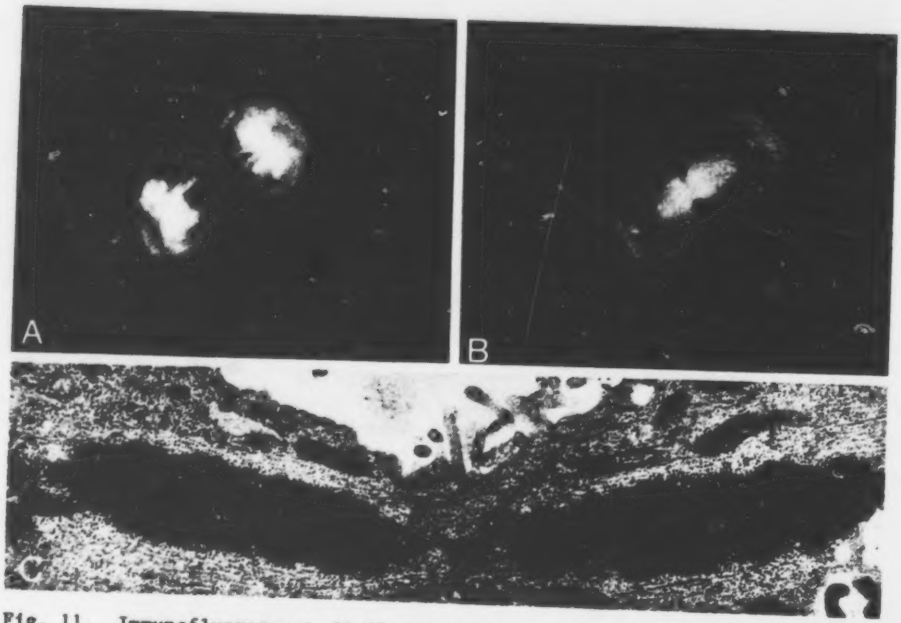

Fig. 11. Immunofluorescent (A,B) and electron microscopic (C) display of lagging daughter chromosomes trapped in the microtubule array of midbody. Micrographs A and B are of the same cell showing chromosomes and spindle, respectively.

Using indirect immunofluorescence, it was possible to evaluate lagging or maloriented chromosomes on the basis of MTs and kinetochores using both antitubulin and antikinetochore antibodies. As shown in Table 2, most of the lagging chromosomes contained kinetochores as indicated by fluorescent staining using scleroderma CREST antiserum. Using a double staining procedure involving Hoescht for DNA and CREST for kinetochores and the appropriate exciter filters, it was possible to observe chromosomes and kinetochores in the same field (Fig. 12,13,14,16). Perhaps the most informative images involved cells double-stained for MTs and DNA. In a few instances, lagging or maloriented chromosomes lacked kinetochore staining (Fig. 13 and 14). As shown in Tab. 2, this was rare, and with this treatment, most chromosomes retained kinetochores. As shown in Fig. 16, lagging chromosomes were usually excluded form the main nucleus and formed micronuclei instead. This explains the correlation between anaphase lagging and the number of multinucleated cells as shown in Fig. 15.

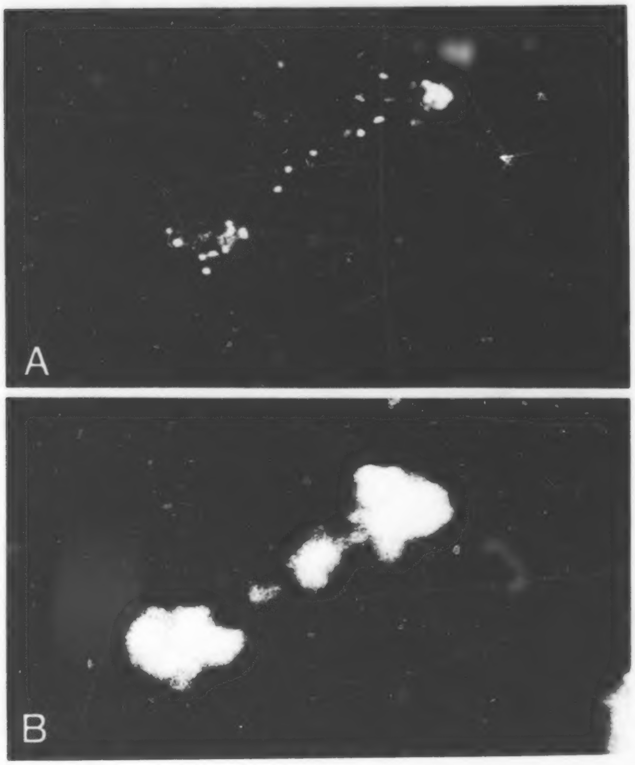

Fig. 12. Telophase Don cell showing lagging chromosomes. Note kinetochores (A) aligned between daughter nucleus. Alignment may be due to spindle microtubules.

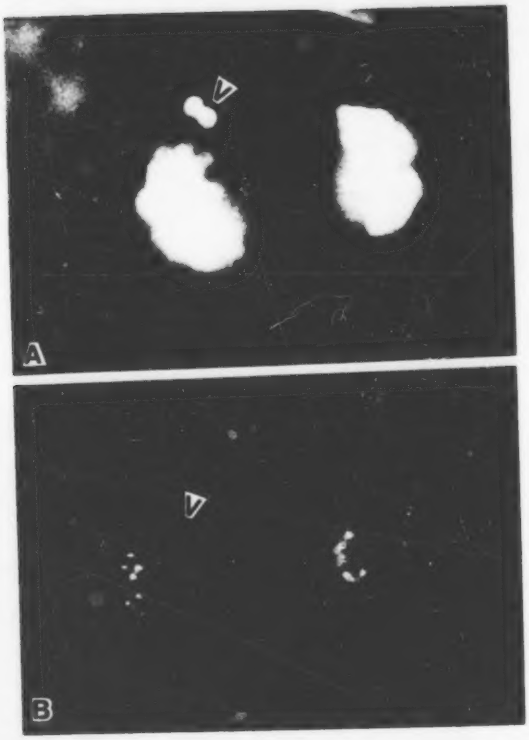

Fig. 13. Double-stained, DES-reversed anaphase cell. (A) Chromatids oriented toward one pole (arrow). (B) Chromatids lack kinetochore staining as shown with CREST immunofluorescence.

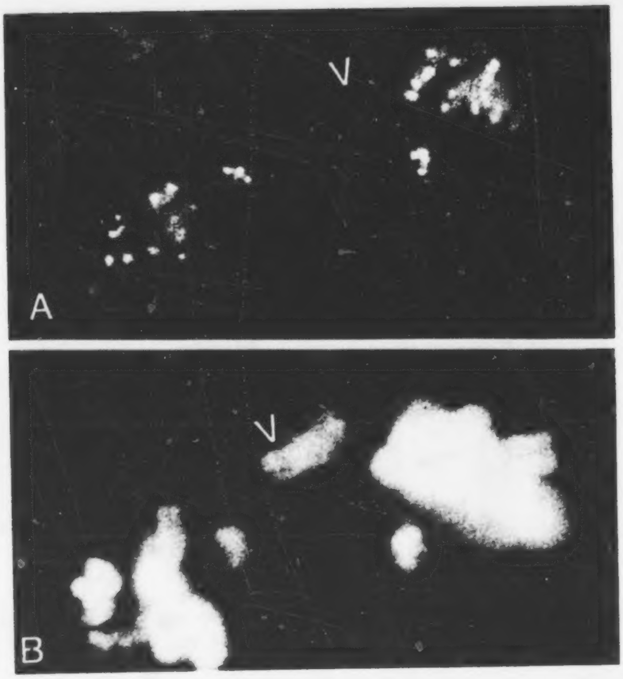

Fig. 14. Double-stained, DES-reversed telophase cells showing lagging chromosomes. (A) CREST antikinetochore stain. (B) Hoescht stain (arrows show absence of kinetochore in lagging chromatids).

SUMMARY AND CONCLUSIONS

The kinetochore is a structurally differentiated site on mitotic chromosomes to which spindle MTs are attached. Although the kinetochore is morphologically distinct from the centromere, the two are functionally linked and necessary for normal anaphase segregation of chromosomes.

The kinetochore is composed of DNA, protein, and possibly RNA. Recent biochemical and immunocytochemical studies suggest that the kinetochore may be made up of several polypeptides of which a 17 Kd and 80 Kd protein can

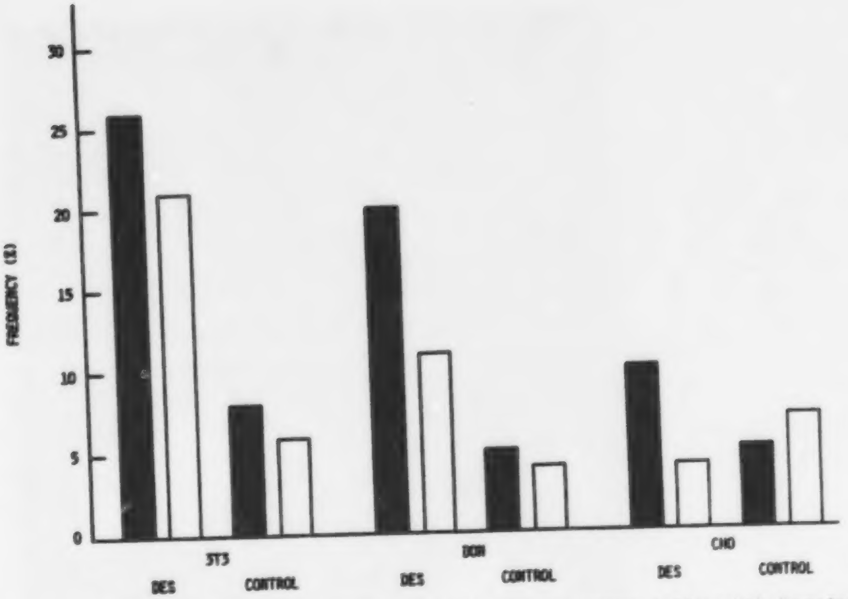

Fig. 15. Histogram showing correlation between anaphase cells with lagging chromosomes (black bars) and multinucleate cells (white bars).

be identified by scleroderma CREST autoantibodies. The latter are specifically located in the kinetochore plates and may be important structural and functional proteins.

Colcemid, a MT inhibitor which binds to 6S tubulin dimer to block MT assembly and a potent inducer of aneuploidy, causes structural rearrangement of the kinetochore. Mitomycin C binds specially with heterochromatin and causes the trilamellar plates of the kinetochore to pull away from metaphase chromosomes.

Diethylstilbestrol, a synthetic estrogen, blocks mammalian cells in mitosis and causes chromosome lagging or malorientation during recovery. Analysis of DES-blocked cells showed unusual chromosome clustering and kinetochore-to-kinetochore MTs. The centrioles were greatly elongated in Chinese hamster Don cells. Lagging or maloriented chromosomes mostly contained a functional kinetochore as indicated by CREST-positive staining and the association with kinetochores. Interkinetochore MTs induced by DES may account for some types of malorientation of chromosomes in anaphase.

On the other hand abnormal chromosome segregation in DES-treated cells appears not to be due to faulty, damaged, or missing kinetochores, or lack of spindle MT association.

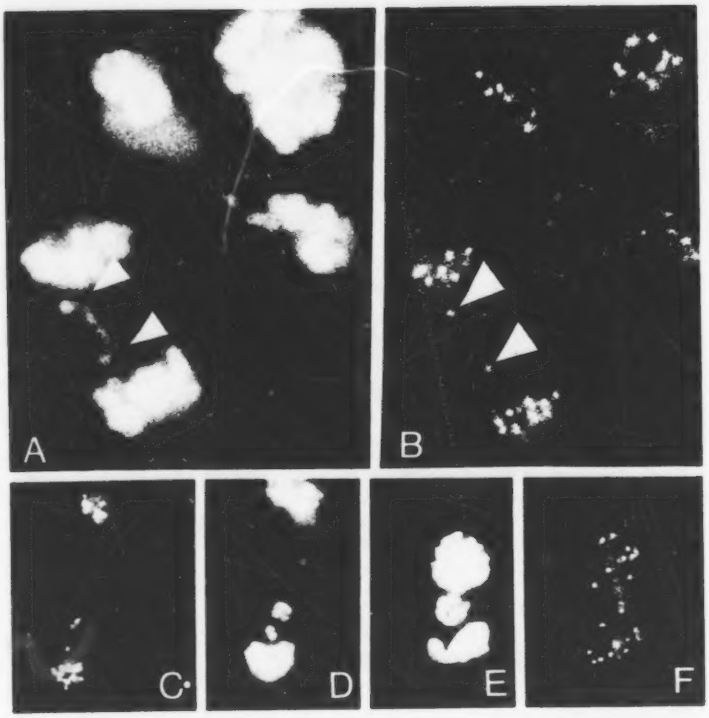

Fig. 16. Lagging chromosome extending between daughter nuclei. Kinetochores (B, arrows) are present. (C-F) Lagging chromosomes become micronuclei in telophase. (C and F) Anti-kinetochore. (D and E) Hoescht stain of same cells.

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DISCUSSION

RESNICK: We have two very different views now on what we call the definition of the centromere: one from yeast and one from mammalian cells. Is it still possible that you could speak of a centromere functional region, which is actually very small like you see in yeast, that would apply to mammalian cells? The amount of DNA that you have described in that slide was almost equal to the amount of DNA in the entire yeast genome, and there may be some very small essential region.

BRINKLEY: I agree that there may be some homologies in centromeric DNA sequences between yeasts and mammalian cells, but to my knowledge they have not been identified. As you know, yeasts have a single MT attached to the centromere at metaphase and no kinetochore plate is seen. Mammals, on the other hand, have a dozen or more MTs attached to the centromere and a kinetochore plate is visible. Apparently a kinetochore plate is only seen when more than one MT interacts with the centromere. In our view, the kinetochore represents proteins and possibly other molecules that interact between MTs and centromeric DNA. When there are sufficient numbers of MTs, we see a plate.

CHERRY: Dr. Hsu and I have been working with a graduate student who has been doing some of our preliminary DES work, and we were very intrigued when he originally found the very narrow dose range at which DES actually seemed to exert a mitotic poison effect. Below it there was no effect; above it it was cytotoxic. So we wondered what the mechanism involved could be, and I was interested to see that you have repeated these findings in Chinese hamster cells and the mouse 3T3 cells. There is a cell line-to-cell line similarity, as well as an interspecies similarity. I was definitely intrigued when I saw a poster from Dr. Barrett and his group at Research Triangle Park in which they have repeated the experiments in Syrian hamster cells and found a linear dose response. I wonder if you have any comments about what sort of differences in mechanisms we might be seeing in these?

BRINKLEY: We do see a very narrow dose range in cells of three species.

BARRETT: Are you talking about for aneuploidy induction or for the mitotic index?

CHERRY: The mitotic index.

BARRETT: We were interested in the effects of aneuploidy-producing chemicals in inducing morphological transformation of early passage cells, which is a first step in a multistep process in neoplastic regression of these cells. We discovered that there was a very marked dose dependence in terms of the cell transformation. Our focus was on how the aneuploidy induction related to the cell transformation induction. One of the interesting findings is that the maximum level of cell transformation occurred at doses below those which caused any effect on the mitotic inhibition. In fact, we find that at higher doses (and it varies from cell line to cell line, which is perhaps not suprising) which arrest cells in mitosis, there is a great deal of polyploidy. Very massive effects. But at lower doses which do not affect cell growth or the mitotic index, you still get a great deal of nondisjunctional-like activity. These are the areas where we see the maximum effects on cell transformation. As a sort of a caution to those interested in looking at effects on aneuploidy, I would think that one should look at some of the lower, as well as some of the higher, doses. What we find is that at low doses (actually down to as low as 300-fold lower than required for induction of mitotic arrest), we still see nondisjunction, which occurs in a linear fashion with a very slow slope. We actually show very similar effects with Colcemid, as well as with DES.

CHERRY: Yes, but you also had a linear response at much higher doses, getting up to about a 50% mitotic index, which is relatively high.

BARRETT: That is very time-dependent. What you find is that the mitotic index goes up with time and then will go down very rapidly as the cells escape this mitotic arrest. And in following that, you will get many cells forming multinucleated cells. So much of that is dependent upon whether or not you did the kinetics as well. That is based upon doing the kinetics first, then doing the dose response at the time where we get the maximum increase with the highest dose. Then you get a dose increase. I do not think it really looks that much different.

PAI: In regards to the one slide you showed where there was a translocation and an inactive centromere. What is the EM structure at that so-called inactive centromere? The kinetochore plates, apparently according to the antibody, are there. Is the EM appearance the same? Are MTs ever attached to that place?

BRINKLEY: First, I would like to make it clear that that is Dr. Hsu's work. We have not been able to section through a region of metaphase chromosomes that contained, beyond a doubt, an inactive kinetochore. It is indeed difficult to preselect sites on chromosomes by light microscopy and section those sites for examination in the electron microscope--especially on human chromosomes.

PAI: There are several patients known who have a supernumerary marker chromosome with dicentric appearance on C-band. One of the C-band regions is split, and it looks like an inactive centromere. Perhaps we could enrich those chromosomes?

BRINKLEY: It would be very interesting to compare the structure and composition of inactive kinetochores of metaphase chromosomes with the "prekinetochores" of interphase nuclei which we know are inactive. But this would be very difficult.

GAULDEN: I have a comment on the mitomycin C effect on separating the kinetochore from the centromere. We have some recent evidence in the grasshopper neuroblast that it is not the centromere actually separated, but rather a physical separation because the chromatids are held together. The centromere action keeps on occurring; the MTs are pulled apart. This is a delayed separation of the sister chromatids with mitomycin C. Now with MNNG, we have shown that at high concentrations you get a real stickiness of the chromosome, and at anaphase, the centromeres separate and go to the poles, leaving most of the chromosomes there. I would like to suggest that perhaps you are not getting an actual separation of the kinetochore, but rather a physical separation.

MICROTUBULE DYNAMICS AND THE MITOTIC CYCLE: A MODEL

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In what follows we will give a personal view of how organized microtubule (MT) systems may evolve during the cell cycle. It is based on some of our own experiments and ideas and on those of many other investigators that have devoted attention to these matters. We will not reiterate the argumentation which has led to the various facets of the following hypothesis, which is based on our personal conjecture. This chapter is similar to the concluding remarks ot a review in press (9). For a detailed account of the data we refer to this review and Ref. 2-8. Figures 1-4 provide the basic illustrations necessary to follow this short review.

During interphase the cell maintains an organized MT system by constructing a "minimal" framework of MTs associated at one end with the centrosome. We propose that this is achieved by a MAP-like protein recognized by the monoclonal antibody, JA2 (10), which is concentrated there. The MAP-like protein functions to lower the critical tubulin concentration (Cc) necessary for assembly and consequently favors MT assembly and protects against disassembly. It also binds MTs more or less tightly by its affinity for the outside wall of the MT. Because this is kinetically the favored configuration, most MTs are associated with the centrosome at their minus end. It is our view that some MTs become firmly attached to the centriolar satellites by their terminal subunits and are thus truly capped (15). These capped MTs are most resistant to inhibitors and may have a different function. A lot has to be learned still about possible molecular differences between various subsets of MTs (e.g., different MAPs, different degree of tyrosylation, etc.) (12) which may have functional consequences. During interphase the cytoplasmic Cc is low, assembly is favored, and much of the available tubulin is in the polymeric form. Free MTs do occur to a variable degree. Because free MTs are dismantled only through detraction of subunits by organized MTs (in cells from which the centrosomes are removed, free MTs are stable; De Brabander et al., unpubl. observ. and Ref. 13), the distance from the centrosome is an important factor. In particular, in large flat cells many free MTs are present most obviously in the cell periphery (9,13). They are simply too far away from the centrosome to be influenced by it. The presence of free MTs superimposed on an organized network does not need to be disturbing to the cells' physiology at all. Actually it would not be too astonishing to find that some MT-dependent functions are driven by free MT fragments sliding along organized MTs.

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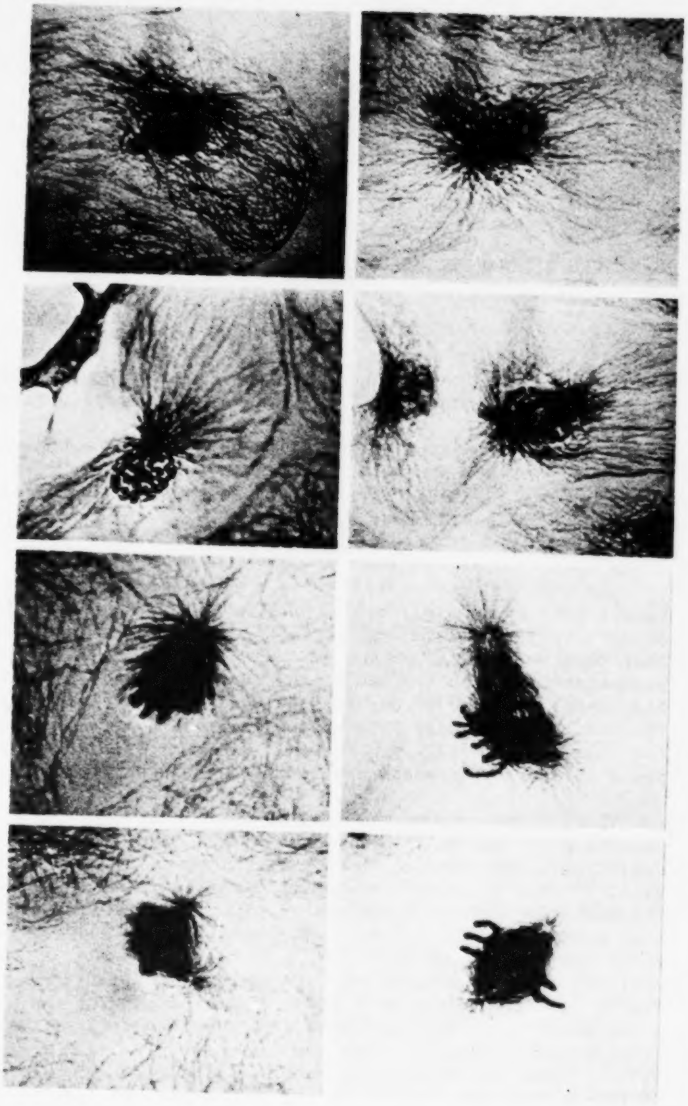

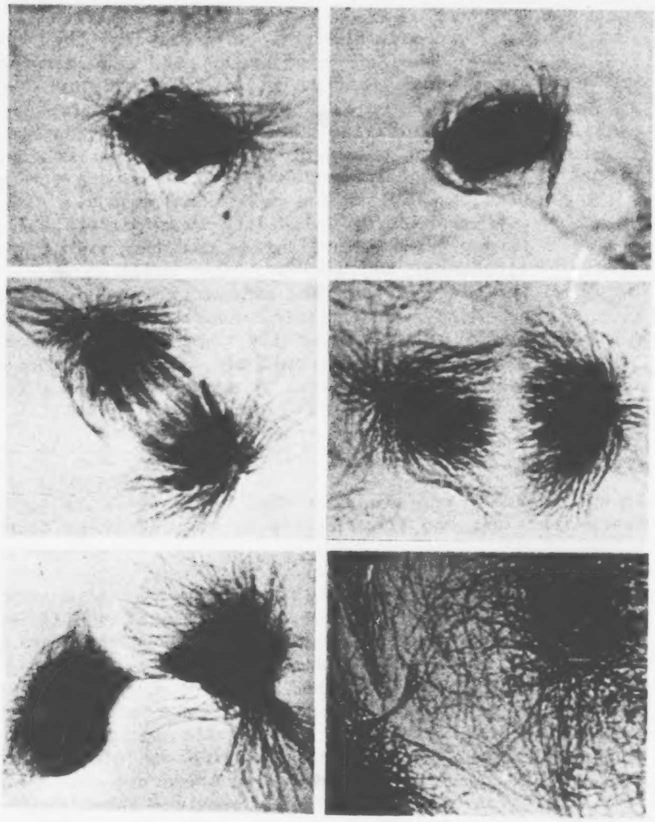

Fig. 1. The turnover of the MT system in PTK$_2$ cells as demonstrated by the staining of the network by an affinity-purified antibody and immunogold (IGS) staining (11) related to the chromatin cycle as demonstrated by toluidine blue staining in the same cells. During interphase most MTs seem to be connected to a focal point in the nuclear vicinity. At prophase the centrosomes have duplicated and migrate either early or late around the disintegrating nuclear membranes. In these cells a central spindle between the 2 centrosomes is prominently absent (see Ref. 18 for an early and detailed ultrastructural account). At prometaphase, whether the centrosomes have already separated or not, the essential change appears to be a shift from assembly of MTs along all radial axes focusing onto the centrosomes, to assembly predominating in the region between the centrosomes and the now structurally, and maybe functionally, ripened kinetochores (18) residing on the centromere regions of the chromosomes. Assembly between the 2 centrosomes, forming a central spindle, is much less obvious. We know that at this stage the kinetochores acquire the capacity to induce MT assembly (3-7,21), an observation which is in current fashion regarded as an experimental curiosity (17). We think it is an essential event in constructing the kinetochore fiber (7).

(continued)

Fig. 1 caption, continued

Two opposing forces, one driving the poles away from the chromosomes (MT elongation) and one drawing the poles to the chromosomes (active sliding of kinetochore-nucleated MT along pole-nucleated MT), are balanced at metaphase (see Ref. 16 for a premonitious view). When the chromatids split at anaphase, the forces are released. The poles migrate in opposite directions while the chromosomes are drawn toward the poles. By an as yet unknown coupling the cell knows that the time has come to form a cleavage furrow coinciding exactly with the equatorial plane of the spindle. Microtubules coalesce and/or grow into an enigmatic structure, the midbody, that still connects the 2 sister cells well into G_1 when the chromatin has decondensed and the nucleolus has been reconstructed. About 1 to 2 hr after careful separation of the equally carefully reduplicated chromatin, this connection will finally be pinched off and 2 new cells will have gained independence, modulated by social intercellular contacts.

Except possibly for the free MTs, the network is rather stable, turnover rates are slow, and treadmilling is not likely to occur to an appreciable extent (9,20).

During prophase the cell prepares itself in a coordinated way for the basic event of life: careful segregation of duplicated genes so that the cell can make a copy of itself. Nature selected an almost failsafe MT-based mechanism to allow the eukaryotic cell to handle more and more complex genetic libraries: the chromosomes. A large variety of systems exists, but we will limit ourselves to the most complex cells: vertebrate ones. In these organisms, the mitotic spindle is not different in its essential parts from the meiotic spindle. Clearly, the correct assembly and functioning of both types of spindles are indispensable for the accurate separation of the chromatids or paired chromosomes, and thus for maintaining euploidy in somatic and germ line cells. Efforts to understand the details of spindle assembly are obviously essential to further the knowledge of the etiology and mechanisms of aneuploidy.

Already when the chromosomes start to condense the MT system is changing its display and turnover rate. Interphase MTs are gradually shortened while new MTs start to grow from the centrosome. The obvious consequence of this rapid assembly process is a greatly increased sensitivity of the system to inhibitors of polymerization (9,19). The system is tightly coordinated probably by the release of an inhibitor of association from the nucleus which increases the Cc in the cytoplasm and leads to disassembly. The released subunits are used to build new MTs from the centrosome, the activity of which, as a nucleating site, is raised by the increased accumulation of a special MAP (10). Interestingly, true capping structures (satellites) disappear at this stage, thus disposing of the most stable class of MT. The difference in the centrosomal Cc and the cytoplasmic Cc is now much greater than during interphase. As a physiological consequence MT assembly is more strictly limited to the microtubule organizing center (MTOC) and the turnover rate of the MT becomes very fast (9,20). Treadmilling may now occur, not driven by the intrinsic polarity, but by the disparity in the Cc at the centrosome and in the cytoplasm.

The rapid growth of MTs from the duplicated centrosomes is probably at least one mechanism whereby they are forced to separate. This is not done by specific interactions of pole-to-pole MTs forming a spindle, but by non-

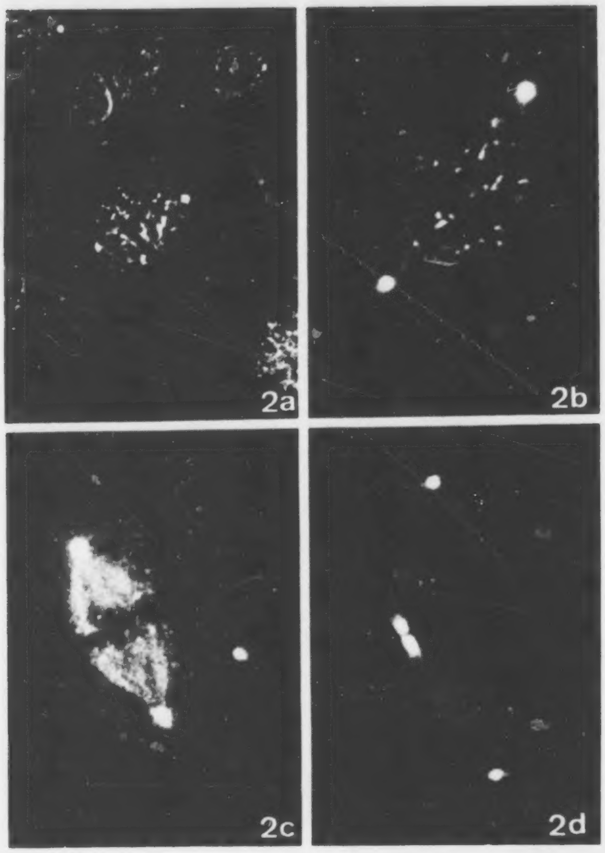

Fig. 2. Immunofluorescent localization of the monoclonal antibody, JA2, that reacts with brain MAP1 in PTK$_2$ cells at different stages of mitosis. Note staining of the centrosome throughout the cell cycle with an increase of intensity during mitosis. The diffuse spots seen in interphase nuclei (a) become clearly double spots located at the kinetochores at late prophase to early prometaphase (b). Kinetochore staining persists throughout metaphase (c). Kinetochore fibers are also weakly stained at this stage. At telophase (d), the central ends of the midbody MT are stained, with the exception of the central plate itself.

specific, growth-related pushing against each other and against the matrix. Keith Porter once explained it to us as 2 cytoplasts which have become mutually exclusive (K. Porter, pers. comm.).

At prometaphase, the Cc in the cytoplasm is at its maximum and the centrosomes drive assembly against this inhibitory influence. By this time the kinetochores have developed into ultrastructurally recognizable entities consisting of the trilaminar plates. Concomitantly, they have accumulated a similar MAP, as is seen around the centrosomes, to such an extent that they override the high Cc and start to induce MT nucleation. These MTs are grabbed by the kinetochore by the minus ends making the kinetochore

MTs intrinsically the least stable ones. They can persist and grow whenever their peripheral end is stabilized by being close enough to the centrosome or by being surrounded by pole MTs. Bringing the chromosomes and centrosomes in close proximity is certainly helped by the prometaphase rounding of the cell which is so uniformly typical for vertebrate mitosis in general. Moreover, this ensures that the whole cytoplasm is close to an MTOC which obviates the inappropriate formation of free MTs in the cell periphery (see above). The process ensures that the kinetochore fiber consists of overlapping kinetochore and pole MTs which have, nevertheless, the same polarity. The latter fact may be important to allow sliding of MTs to occur. In cilia and flagella dynein-mediated sliding also occurs between MTs of the same polarity.

Growth of MTs from the poles has now also become polarized. Most MTs grow towards the chromosomes indicating that they, or some part of them (centromeres or kinetochores), have a stabilizing influence on MTs growing

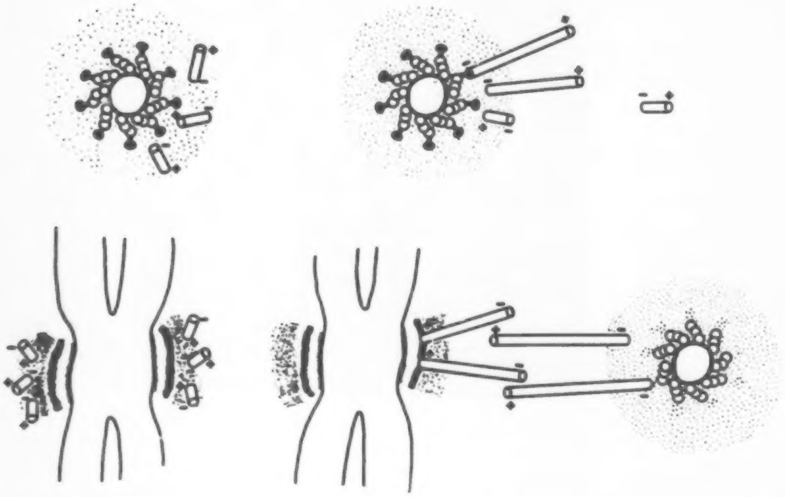

Fig. 3. Some aspects of our model for MTOC activity. The centrosome and kinetochore are surrounded by material (MAP-like?) that lowers the Cc. The left schemes depict the situation after release from nocodazole-induced disassembly. New MTs are nucleated preferentially in the vicinity of the MTOCs. At steady state (right panel) some MTs have been anchored to centriolar satellites, while other MTs merely maintain their minus end in the assembly-promoting environment. Because the free plus ends incorporate subunits until the tubulin concentration has dropped to Cc^+, free MTs or MTs growing from the MTOC with their minus end free must disassemble gradually. During mitosis (lower right) kinetochore MTs become anchored with their plus ends in the kinetochore plate. The peripheral minus ends can only grow or persist if one assumes a stabilizing influence arising from the vicinity of the centrosome or from the interaction with centrosome-derived MTs. Additionally, new subunits may be incorporated at the kinetochore-associated end at a rate faster than the subunit loss at the peripheral end.

from the centrosome (see Ref. 14 for recent experiments along these lines). The sliding of the 2 sets of kinetochore fiber MTs draws the chromosomes towards the poles. Because each chromosome has 2 kinetochores this results in a shuffling motion between the two poles. Of course this also results in both poles being drawn towards the chromosomes (prometaphase shortening of the spindle). Probably the continuous growth of MTs (kinetochore and interzonal MTs) drives the poles away from the chromosomes and thus results in the final construction of the bipolar metaphase spindle when the opposing forces are balanced. During this stage, many short, free MTs exist within the spindle area. These may be breakdown products, or they may just show that, in the region between the poles and the chromosomes, the Cc is indeed much lower than in the peripheral cytoplasm. Whether treadmilling occurs at this stage is a matter both of debate and of active investigation.

At this point it is useful to highlight a few elements which are of particular relevance to the maintenance of euploidy. The correct assembly of the kinetochore fibers appears to be the single most important event necessary for the accurate construction of the bipolar spindle and for subsequent equal separation of the chromatids. Most of the time of mitosis is used for this process: prometaphase and metaphase take more than 70% of the total time. It appears as if this is necessary to allow ample time for trial and error. As has often been stated (e.g., Nicklas, this Volume), the structures also show much redundancy. Moreover, several processes which do not appear to be essential for mitosis may have a supportive role in favoring kinetochore- and centrosome-associated assembly instead of free assembly (e.g., the general increase in the Cc and cell rounding). Additional supportive mechanisms probably exist, but are as yet undiscovered. Finally, the general scheme that we propose implies that the chromosomes are themselves the major governors of the correct assembly of the spindle (and in particular the kinetochore fibers) and of the correct timing (see Ref. 7 and 9 for a more elaborate discussion). In vertebrate cells chromosomes do not merely attach to a preformed spindle, unlike what may be the case in less complex organisms, such as diatoms (17). Teleologically, this makes good sense; it is more important for complex organisms, containing complex sets of genetic libraries, to maintain euploidy than it is for unicellular protozoa.

Chromatid splitting at anaphase results in release of both forces to produce persistent motion. The chromatids slide towards the poles while the MTs forming the kinetochore fiber shorten. The poles move apart by

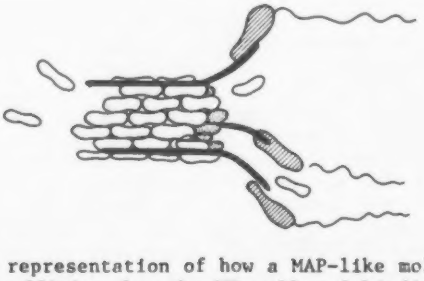

Fig. 4. Schematic representation of how a MAP-like molecule (dark lines) having an affinity for the MT wall and binding sites for structural components of the MTOC may bind MTs without blocking association or dissociation of subunits. By strengthening longitudinal bonds, however, the molecule decreases the dissociation rate, and thus the Cc.

further growth of the interzonal MTs which incorporate subunits derived from the shortening kinetochore MTs. Sliding of interzonal MTs of opposite polarity is in our view, and that of others (1), of no or little importance as a force producer during anaphase in the mammalian spindle. Rather, they may act as governors of speed and direction. Moreover, the overlapping interzonal MTs at late anaphase serve as a substrate for the accumulation of the dense material that will finally become concentrated in the Flemming body (midbody). Apparently, this material is partly similar to that present previously on the kinetochore and centrosomes: a MAP-like protein that stabilizes MTs (10). The anaphase spindle thus determines the site where the constriction will appear. Maybe the removal of this MAP from the cytoplasm and/or the shift of this protein from the kinetochores and centrosomes to the forming midbody, serves as a trigger that starts the clock which times and coordinates various other telophase events. The cleavage furrow separates the daughter cells, the chromatin decondenses while a new nuclear membrane is zipped onto it, and Golgi elements assume again an organized aspect. Later on the cells flatten again, actually crawl onto the substratum, and assume gradually their normal interphase shape.

Conditions for microtubule assembly and turnover also change drastically during this period. The Cc in the cytoplasm decreases gradually to the interphase level. This is not due to a constitutional degradation rate of the putative endogenous inhibitor, but through a timed removal or degradation coordinated by the telophase trigger. At the same time the MTOCs change. The kinetochores have lost their nucleating power probably during anaphase. More experimentation is needed here. The centrosomes lose much, but not all, of their nucleating activity. The satellites reappear and gradually a typical interphase network is built from the subunits released from the disintegrated spindle. During its construction this network is still highly susceptible to inhibitors of assembly, but as soon as equilibrium is reached the typical interphase turnover rate and stability are adapted. Although much of this interpretive description may turn out to be incorrect, we hope, nevertheless, it may serve a useful purpose as a matter of discussion and framework for future experimentation.

ACKNOWLEDGEMENTS

We are indebted to L. Leijssen and G. Jacobs for photography and to B. Wouters for secretarial work. We wish to acknowledge the gifts of antibodies from J.V. Kilmartin, V. Kalnins, E. Tan, and G. Warren. Supported by a grant from the IWONL, Brussels, Belgium.

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DISCUSSION

RISLEY: What is known about the interaction of the centrosome, or more specifically, the centriole, with the nucleus prior to nuclear membrane breakdown? A number of cytological observations suggest that organization within the nucleus may be very strongly related to the positioning of the centriole prior to nuclear membrane breakdown. What do we know about the structural interactions that go on there?

BRINKLEY: Several laboratories have noted that if you isolate nuclei you often pull out the centrosome and centrioles. Some believe this association is due to the presence of intermediate filaments in that region (intermediate filaments may entrap the centrosome). There are older reports that suggest some sort of functional connection between the centrosomes and the kinetochore during interphase, but I believe such interactions have not been found.

MCINTOSH: One of the properties you described in the centrosome was that the number of MTs that initiated would not be defined. But I think there are a couple of studies which suggest that it is defined. Do you disagree with those studies, or do you relate them to your model in a different way?

DE BRABANDER: If you do a certain experiment and have regrowth, then start to count MTs, you can have a median value. But there is quite some variability.

MCINTOSH: In experiments by Goll and by Mitchison and Kirschner, centrosomes were isolated (MT-free) and put in different concentrations of MT protein. The number of MTs which grew was proportional to the tubulin concentration up to a saturating level. The saturating level was 50 or so for a mammalian centrosome from an interphase cell, a number below what you could expect for a sphere of MTs closely packed. Yet it is a defined number, suggesting that there are defined loci that nucleate the MT of the centrosome.

DE BRABANDER: Yet those same centrosomes in vivo have many more than 50 MTs associated with them.

BRINKLEY: Mitchison and Kirschner (Nature) showed that isolated centrosomes become saturated with MTs after a period of incubation with pure tubulin above the critical concentration. We reported the same effect in permeablized cells. These results argue that MT organizing centers generate a finite number of MTs. We also see evidence for this in living cells. Working with Don Anderson at Baylor, we found that human PMNs display a set of 34 MTs/cell and retain this number during anchorage, orientation, and movement. MTOCs can count and we think this is important in MT function.

GAULDEN: I would like to comment on the orientation of the chromosomes in the nucleus. We have some recent data with mitomycin C indicating that in the grasshopper neuroblast, which is about 30 μ in diameter with a big nucleus, the chromosomes are attached to the nuclear membrane at the centromere region. We can put a microneedle into that nucleus and move the distal portions of the chromosomes in the untreated cells, and you cannot dislodge them from the nuclear envelope without a great deal of force. Years ago we showed with high concentrations of colchicine that they would be blocked; some in very late prophase would just sit there for a long period of time. The chromosomes would lose their orientation. We thought that this was because the colchicine was interfering with the centriole and the attachment up around the nucleus. We still get the same result. Mitomycin C in our cells has absolutely no effect at all on MTs and the centrioles. I think the orientation is maintained by an interaction of the chromosomes and attachment to the lamens on the inner surface of the nuclear membrane. I think the mitomycin C is interfering with them, and that may be one of the factors that is controlling orientation.

ANEUPLOIDY INDUCTION BY MITOTIC ARRESTANTS IN ANIMAL CELL SYSTEMS: POSSIBLE MECHANISMS*

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INTRODUCTION

It should be emphasized at the beginning that the mechanisms for aneuploidy production can be quite different between meiotic divisions and mitotic divisions. A translocation heterozygote can produce nondisjunction (hence aneuploid gametes) in meiosis but not in somatic cells. Thus, in spermatogonia or first meiosis a mutagen (or a clastogen) can induce aneuploid gametes by first inducing rearrangements, but such agents rarely induce aneuploidy in mitotic cells because, as far as the number of centromeres is concerned, rearrangements (except in cases of Robertsonian fusion) do not alter the number of centromeres.

Aneuploidy (such as the result of a nondisjunction) means a gain or loss of a chromosome(s) of a genome. Therefore, it is suggestive that an error occurs in the cell division mechanism. The target cellular structures for agents that can induce aneuploidy should be those which are employed by cells to ensure proper distribution of chromosomes into daughter cells, not DNA. Several such organelles or events are involved:

(a) The spindle apparatus to which the chromosomes are attached.

(b) The centrosomes that migrate to the poles during mitosis to serve as spindle organizing centers.

(c) The kinetochores (centromeres) that attach the chromosomes to the spindle.

(d) The cytokinetic mechanism by which a mother cell divides into 2 daughter cells.

As will be seen, all these structures and events are interrelated and coordinated; but for convenience, we shall use the term, mitotic poison, to cover all agents that can interfere with the course of orderly cell divi-

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* Supported in part by Research Grant R-808582 from the U.S. Environmental Protection Agency.

sion. However, one group of mitotic poisons, the mitotic arrestants, has received the most attention. Therefore, our discussion will be devoted more to this group.

A comment should be added at the beginning that it is unlikely that all mitotic poisons can induce aneuploidy. For example, if an agent causes only the disruption of cytokinesis but not karyokinesis, then the resulting cell will be a binucleated or a polyploid cell because chromosome separation is presumably not affected.

MITOTIC ARREST

The first mitotic arrestant found, and still the most well-known, was colchicine (6). Colchicine is an alkaloid extracted from the plant, Colchicum. Colchicine inhibits the formation of the spindle apparatus without which the mitotic chromosomes cannot disjunct and move to the poles. Therefore, cells entering mitosis in the presence of colchicine are blocked at metaphase. Usually, the chromosomes are clumped in the center of the cell (c-mitosis) with no anaphase movement. However, the chromosomes continue to condense and eventually the sister chromatids separate, including the centromeres; but due to the lack of the spindle apparatus, sister chromatids lie side by side (c-anaphase). The chromatids finally despiralize as if they were in telophase (c-telophase), and a diploid cell enters the next interphase as a tetraploid. Superficially, almost all mitotic arrestants, such as colchicine, Vinca alkaloids, diazepam, diethylstilbestrol (DES), etc., induce similar cytological effects (c-mitosis). The underlying mechanisms and details, however, may be different, but such differences cannot be detected without electron microscopy and fluorescence microscopy using antibodies against several cellular organelles. Taxol, another alkaloid produced by the plant, Taxus (17), induces mitotic arrest with a strikingly different cytological picture: the metaphase chromosomes, instead of aggregating in the center, are dispersed at the periphery of the cell, so that they are individually visible in ordinary squash preparations without the use of a hypotonic solution (14).

Most cytogeneticists use mitotic arrestants to accumulate metaphase plates for the purpose of analyzing chromosomes, and most cytologists study the effects of mitotic arrestants on cells by a continuous treatment. However, the phenomenon of recovery, i.e., overcoming the arrest after removal of the drugs in the substrate, is an integral part of mitotic poisoning. We have found that recovery potential is different among various drugs, and errors in recovery may play a major role in aneuploidy induction (14).

Again, we may use colchicine or Colcemid as an example. In cell cultures, if the growth medium containing the agent is replaced by drug-free medium, recovery readily takes place. In Chinese hamster cells, a normal metaphase plate can be formed within 10-15 min following removal of colchicine, and anaphase begins. In 30-45 min, practically all blocked metaphases complete cell division.

All mitotic arrestants react with the cellular protein, tubulin, and its polymerized cellular structures, microtubules (MTs), in one manner or another. MTs constitute the skeleton of a cell as well as the principal component of the asters and the spindle apparatus. Normally, tubulin polymerizes to form MTs, which then depolymerize to tubulin molecules in equilibrium. When a cell enters mitosis, the equilibrium is disrupted. Most MTs depolymerize to tubulin and repolymerize to form the asters and the spindle. Colchicine and Colcemid prevent the polymerization process, but do not interfere with the depolymerization process. From a detailed electron microscopic study on the effects of Colcemid on mitosis in Chinese hamster cells, Brinkley et al. (4) found that in arrested metaphases, the two pairs of centrioles, which normally migrate to the poles to organize the spindle, were situated in the center of the cell with only a scanty number of MTs emanating from them. The chromosomes were arranged at some distance from the center. The kinetochore of one sister chromatid of each chromosome faced the center with a few MTs connecting it and the centriole, while the other sister chromatid faced the plasma membrane with a bare kinetochore.

Colchicine does not interfere with growth of cells, so that cycling cells continue to enter mitosis, but are then blocked. Therefore, metaphase accumulation becomes a most dominant cytological phenomenon.

When Colcemid is removed from the medium, recovery quickly ensues. The two pairs of centrioles migrate away from each other and the chromosomes reorient their positions to form a metaphase plate with MTs emanating from the kinetochores to the poles. Anaphase movement follows. Thus, when colchicine is removed, polymerization of tubulin can promptly take place with apparently no ill-effects.

MECHANISMS OF MITOTIC ARREST

Different mitotic arrestants have different reactions with tubulin and MTs. The following types have been known.

Colchicine Type

Colchicine, or its synthetic derivative, Colcemid, prevents tubulin polymerization. These agents do not interfere with MTs in existence. Therefore, they do not interfere with mitotic cells already at metaphase or anaphase. When the agent is removed, recovery (spindle reorganization followed by anaphase movement) is usually prompt.

As far as the mode of action is concerned, probably most mitotic arrestants belong to the colchicine group: interfering with polymerization but not depolymerization. Anderson et al. (1), in a study on the effects of diazepam (Valium), showed that metaphases arrested by diazepam had centrosomes situated in the center of the cell and the chromosomes surrounding this center. These authors postulated that diazepam inhibits the separation of centrioles, hence arresting mitosis at premetaphase. From gross appearance, the diazepam-arrested mitosis is no different from those arrested by Colcemid. Although no electron microscopic observations have been made in diazepam-induced arrest, we believe that diazepam probably also exerts its poison effects by interfering with tubulin polymerization.

Hypothermia

Hypothermia has long been known as an agent that can disrupt mitotic spindles. Unlike colchicine, which does not destroy MTs already in existence, hypothermia action is indiscriminative. Since hypothermia retards all biochemical reactions of the cell, few cells in cycle will enter mitosis after the chill. Therefore, there is no metaphase accumulation by hypothermia. However, recovery can quickly take place following a return of cells to incubation temperature.

Vinca Alkaloids

Vinblastine and vincristine are two well-known Vinca alkaloids. Superficially, the effects of Vinca alkaloids on cell division are not different from those of colchicine, i.e., mitotic arrest and accumulation; but their mechanism of action is unique. At higher concentrations, e.g., 10^{-5} M or higher, these compounds precipitate all available tubulin and MTs of the cells into paracrystals (2). Such cells, when arriving at mitosis, are unable to organize a spindle apparatus and are therefore arrested at metaphase. These cells cannot recover, even when the agents are removed from the substrate, because free tubulin molecules are no longer available. The arrested cells remain arrested for long periods of time, eventually entering interphase without anaphase movement, and become polyploid cells.

At lower concentrations, Vinca alkaloids do not induce tubulin crystals, but still arrest mitosis. It has been postulated that there may be two different mechanisms, i.e., at lower concentrations, Vinca alkaloids behave in a way similar to colchicine (3). Indeed, at lower concentrations, such as 10^{-7} or 10^{-8} M, anaphase recovery can be readily observed in cell cultures.

Taxol

The reaction of taxol to tubulin and MTs is the opposite of colchicine, i.e., it permits tubulin polymerization to form MTs, but inhibits depolymerization of MTs to tubulin molecules (15). When a cell is exposed to taxol for a period of time, all available tubulin molecules will be used to form MTs. Therefore, the cytoplasm will be filled with extensive bundles of MTs. But when this cell enters mitosis, there will be no tubulin left to form a spindle apparatus, hence metaphase arrest.

MITOTIC ANOMALIES IN RECOVERING ANAPHASES

Stubblefield (16) found, in cinematographic records, that Chinese hamster cells arrested by Colcemid would exhibit mitotic abnormalities (such as tripolar and tetrapolar anaphases) following recovery, and that the frequency of abnormal anaphases increased when the arrested period prolonged. Recently, we confirmed this observation using cytological preparations for obtaining more quantitative data (7,8,14). By treating cultured Chinese hamster cells with several mitotic arrestants (Colcemid, vinblastine, diazepam, DES, and taxol) to arrest mitosis and then reversing the arrest by removing the drug from the medium, our experiments provided the following general conclusions:

(a) Different arrestants have different potency. To achieve complete metaphase arrest by diazepam or DES, a concentration of 10^{-4} M is necessary while the same effect can be obtained by other agents at 10^{-7} M.

(b) Reversal of arrest by diazepam, DES, and Colcemid can be accomplished at any concentration, but no reversal is possible with vinblastine or taxol at 10^{-6} M or higher. With vinblastine or taxol, reversal is possible when the concentration is $\leq 10^{-7}$ M.

(c) In untreated cell populations of the Chinese hamster cell line used (Don), the frequency of abnormal anaphases (lagging chromosomes, multipolar spindle) seldom exceeds 2%. After mitotic arrest and reversal, however, the frequency may reach as high as 25%, depending upon the duration of arrest. Generally, longer periods of arrest induce a higher frequency of abnormal anaphases during recovery.

(d) The frequency of aneuploidy in cell populations 18-24 hr following recovery of metaphase arrest is also drastically increased, from the control level of 1-2% to around 20%. This observation

> confirms and extends the earlier conclusion of Kato and Yosida (10) who worked on Colcemid-induced aneuploidy using the same cell line.

Anomalous cell divisions are not limited in cells recovering from metaphase block. Ostergren and Levan (13) noted in plant meristems that when drug concentrations used were not sufficient to arrest all mitoses, some cells might have slipped into anaphase. Among these anaphases, abnormalities were present. More recently, Liang et al. (12) recorded the same phenomenon in grasshopper embryo cells exposed to benzene. Although no data on chromosome counts accompanied these experiments, we have little doubt that aneuploid cells were generated. Indeed, in Chinese hamster cells, Cox and Puck (5) reported that Colcemid, at concentrations not effective to induce mitotic arrest, induced aneuploidy.

If an arrested diploid cell does not reverse, it will eventually become a tetraploid cell. There will be no genetic imbalance. It therefore appears that the damage to the cellular genome by mitotic arrestants lies in two phenomena: (a) anaphase recovery and (b) incomplete arrest.

Although we do not have extensive information regarding the relationship between mitotic arrest and aneuploidy induction, we are confident to predict that any agent capable of arresting mitosis is capable of inducing aneuploidy. Therefore, the assay system can be simplified by merely testing an agent's capability of mitotic arrest and accumulation. If an agent proves to be a mitotic arrestant, it is almost certain that it will also produce aneuploidy. One caution should be made. If an agent is a fixative, such as formaldehyde, many acids, etc., such an agent may fix the cell population when the concentration reaches a certain level. In such cases, cytological preparations will give an impression that the cells are perfectly normal. A wide range of concentrations, perhaps down to 10^{-8} or 10^{-9} M, may be necessary to test the effects of these compounds on cellular structures and behavior.

We have been using 3 test systems for mitotic arrestants: mammalian cells in culture, grasshopper embryos in ovo, and bone marrow cells in vivo. Detailed descriptions for these systems are presented in a review (9). In essence, all substances soluble in water or dimethylsulfoxide (DMSO) can be tested with cultured mammalian cells. The chemical should be tested with a minimum of 4 concentrations: 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M. A treatment time of 5 hr should be sufficient. Two parameters should be obtained from cytological preparations (standard air-dry or squash) made from these samples: the mitotic index and the anaphase:metaphase ratio (A/M). Comparing data from the untreated control and the carrier control with those of treated samples should reveal whether there is an accumulation of metaphases and a reduction or absence of anaphases. If both are positive, the agent is a mitotic arrestant.

Grasshopper embryos (12) are excellent for testing vapors of volatile liquids. Embryos can be exposed in sealed containers, and at a desired exposure time, they can be fixed for squash preparations for reading the mitotic index and the A/M ratio. Bone marrow of laboratory animals, such as mice or Chinese hamsters, can be effectively employed for the same purpose (11).

POSSIBLE MECHANISM FOR ABNORMAL MITOSES

Is there really such a phenomenon called nondisjunction? If so, what causes it? What causes chromosomes to lag? What causes multipolar spindles? Nondisjunction (failure of sister chromatids to move to separate poles during anaphase) and trisomy or multisomy have been almost synonyms. Prior to the present report, nondisjunction was primarily inferred, not cytologically observed. In our material (Fig. 2e) nondisjunction during anaphase is common, yet accurate quantitative data are difficult to obtain.

A working hypothesis may be briefly described as follows. Normally, the chromosomes in interphase are encased in the nucleus. Exchange between the nucleus and the cytoplasm is limited through the pores of the nuclear envelope. Presumably, chromosomes in the nucleus have little or no contact with many cellular enzymes in the cytoplasm, such as proteolytic enzymes and nucleases. The only time at which the chromosomes are directly exposed to the cytoplasm is the brief period when the cells reach metaphase and anaphase. When mitotic cells are arrested at metaphase, the exposure time of the chromosomes to the cytoplasm is artificially prolonged. Thus, an interaction between chromosome and cytoplasm is also prolonged, and cytoplasmic enzymes may have an opportunity to react with proteinaceous structures of the chromosomes, including kinetochores. If the kinetochores of a chromosome are damaged or destroyed by proteases, then this chromosome cannot attach itself to the spindle apparatus when the arrested cell finally recovers to reorganize a spindle. Therefore, this chromosome will become a lagging chromosome. If the kinetochore of a sister chromatid is damaged while that of the other sister is not, nondisjunction may be the result.

In collaboration with Dr. B.R. Brinkley, to test whether this hypothesis of kinetochore damage has any validity, we used an antikinetochore serum from a scleroderma patient for indirect fluorescence microscopy. Limited data obtained by Brinkley and his associates discovered that, while poleward chromosomes exhibited fluorescence at the centromeric area, some lagging chromosomes showed no demonstrable fluorescence, suggestive of damaged or missing kinetochores. From one set of serial sections, electron micrography failed to reveal any trace of a kinetochore in a lagging chromosome. Of course, many more data are needed; but the approach appears to be useful to probe the mechanisms of abnormal mitosis and, consequently, aneuploidy induction. If the supposition is correct that kinetochores (chromosomal proteins, as well as centrosomes) are vulnerable to proteolytic enzyme digestion, then artificially introducing exogenous proteolytic enzymes into dividing cells should increase the frequency of abnormal mitoses. Our preliminary experiments using both Chinese hamster and Indian muntjac cells seemed to support this contention. After mitotic arrest with Colcemid, the cells were treated for 15 min with a balanced salt solution containing DMSO and either trypsin or pronase. They were then reincubated in regular growth medium for 30 min. There was a marked increase in frequencies of multipolar spindles and lagging chromosomes (Fig. 1 and 2). Our data also showed that trypsin treatment alone resulted in the same level of mitotic abnormalities as trypsin with DMSO, indicating that trypsin molecules can penetrate cell plasma membrane. If our data can be substantiated, then it would appear that routine use of trypsin in cell subculturing may be, however slowly, one of the causes of genomic changes in longterm cell lines.

DISCUSSION AND CONCLUSIONS

It appears reasonably certain to conclude that at least in cell cultures, all agents that can cause mitotic arrest can cause aneuploidy after the cells recover from the block. Whether this conclusion can be applied to cells in vivo, and if so, whether the effects are reduced or enhanced, remain to be tested. Several differences exist between in vitro and in vivo situations, as follows. (a) In cell culture, each cell is a target cell. All cells are directly exposed to the drug and presumably receive equal exposure. Thus, it is relatively accurate to construct dose-response

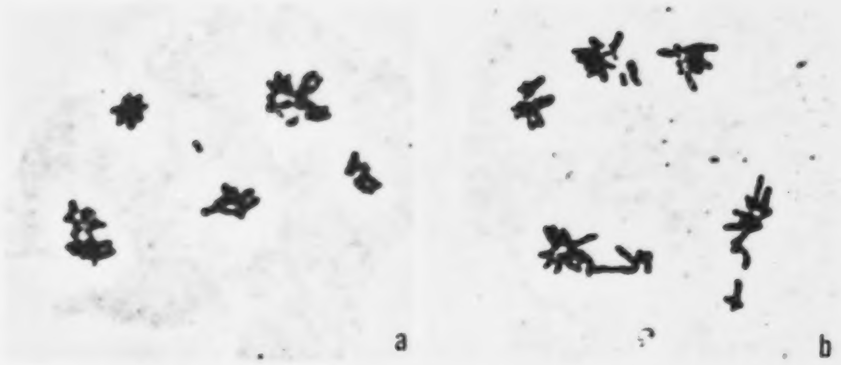

Fig. 1. Multipolar anaphases of Chinese hamster cells. Cultures were pretreated for 2 hr with Colcemid (10^{-7} M), then for 15 min with a buffered solution containing trypsin (0.03%) and DMSO (1%), and reincubated for 30 min in drug-free medium. (a) 5 poles. (b) 6 poles.

curves and other parameters. In experiments in vivo, regardless of the route of administration, all tissues and cells in the organism would absorb the drug. Therefore, the amount of drug molecules finally reaching the target cells should be conceivably less that that injected or fed. In other words, an effective dose of 10^{-6} M for cells in vitro may be ineffective for bone marrow cells in vivo. (b) In cultured cells, metabolic activation or inactivation does not take place unless the cells are cocultivated with hepatocytes or treated with the liver microsomal fraction, S9. In experiments in vivo, metabolic changes of the drug molecule may take place. (c) In cell cultures, when a drug is added to the medium, it remains there until the medium is replaced by a drug-free medium. In the body, various rates of excretion and/or decomposition may take place, but the drug cannot be suddenly depleted. (d) It is not feasible to perform recovery experiments in vivo.

Nevertheless, some inference can be drawn for comparison between the in vitro and the in vivo data. In experiments to test aneuploidy induction by Colcemid and vinblastine in vivo, Liang and Satya-Prakash (11) injected these drugs into Swiss mice and observed the responses of mitotic cells of the bone marrow. These investigators found that at high doses both agents are capable of arresting all mitotic divisions at metaphase, but the frequency of aneuploid cells in cell populations 17-72 hr postinjection was high only in the vinblastine series. The data suggest several pertinent points. (a) Recovery from mitotic arrest did take place in at least some of the cells. (b) Recovery must have been faster in Colcemid-treated than in vinblastine-treated animals. In cell cultures, recovery is prompt after Colcemid arrest, while this is not the case with vinblastine at higher concentrations. Assuming an equal rate of excretion in vivo, recovery probably is also much faster with Colcemid than with vinblastine. This difference may account for the low frequency of aneuploid cells in the bone marrow. (c) Although the frequencies may differ between cells in vitro and in vivo, aneuploidy can be induced by mitotic arrestants in both systems. In a patient treated with taxol, cultured lymphocytes without a pretreatment with a mitotic arrestant exhibited typical c-mitotic appearance and tripolar anaphase (Liang and Savaraj, pers. comm.). Since lymphocytes were stimulated to grow in the absence of taxol, the lingering effect of taxol therapy is obvious.

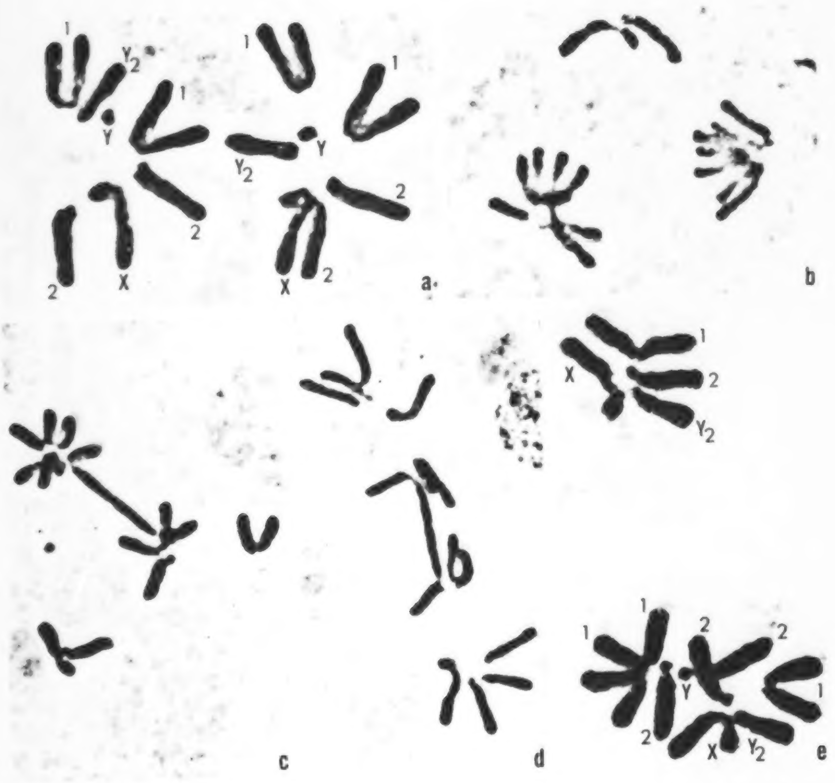

Fig. 2. Anaphases of male Indian muntjac cells similarly treated as the Chinese hamster cells shown in Fig. 1 except that the Colcemid concentration was 10^{-8} M. (a) Normal anaphase; each chromosome can be recognized at this stage. (b) A tripolar spindle. (c) Multipolar spindle with a pseudochromatin bridge. The No. 1 chromosome has a nucleolus organizer region near the tip of its long arm. In anaphase the ends of the sister chromatids may be temporarily held together by the remnant of a nucleolus, thus creating an impression of a chromatin bridge. It is not a true chromatin bridge resulting from a translocation or a dicentric chromosome. (d) Bipolar spindle with a pseudochromatin bridge and 2 laggards. (e) Anaphase showing nondisjunction. Top pole, 4 chromosomes: #1, #2, X, and Y_2; bottom pole: 3 #1, 3 #2, X, and Y_2. The real Y is absent in this cell.

We believe that any chemical that can arrest mitosis has the potential to produce aneuploidy, at least in somatic cells. However, extensive studies must be carried out on the impact of mitotic arrestants on gametogenesis. Moreover, not all known chemicals that can induce aneuploidy are mitotic arrestants. Verification of aneuploidy induction in animal cells and proposing the mechanisms of action of such chemicals must be done.

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DISCUSSION

GENEROSO: It seems that when there are multiple chromosomes involved in nondisjunction, these chromosomes always come from the same set. Have you had the case where two or three chromosomes were involved, but those two chromosomes came from different sets?

HSU: It is very difficult to make a determination the way we do it because we deliberately use the timing to see lagging chromosomes when the cells are in late anaphase. At that time you cannot really count the chromosomes.

SATYA-PRAKASH: We looked at anaphases after blocking the Don cells for 2 hr or 4 hr. We saw a lot of abnormal anaphases. When we looked 24 hr later at the next cell cycle, we found sometimes, in a very low percentage, normal looking diploid metaphases. But when you look carefully at the chromosomes, you can make an eye-karyotype. The cell may be missing an X chromosome and adding an extra chromosome which does not belong to the karyotype. In other words, it was a pseudodiploid.

GENEROSO: So you are saying that it could happen?

SATYA-PRAKASH: Yes, it could happen.

BOND: I was interested to hear that you used DMSO as a vehicle for penetrating into cells, because DMSO itself induces aneuploidy in several systems. Did you have any evidence that DMSO induces aneuploidy?

HSU: We have done quite a few preliminary assays in Chinese hamster cells. The concentration of DMSO we use does not seem to affect mitosis at all. In about a 7-hr run, we assayed every 2 hr, and found that the mitotic behavior of DMSO-treated cells was similar to that of the controls. But at higher concentrations of DMSO, I admit there was a detrimental effect.

WARBURTON: Have you looked at the distribution of the chromosomes which lag? Are there particular chromosomes which lag more often than others? In particular, does this have anything to do with the amount of heterochromatin in the particular chromosomes?

HSU: We just started on this. We will probably be able to give you an answer in 2 or 3 months, but right now I do not have one. As a guess, no. I think any chromosome can lag.

SHARMA: In an industrial set-up, many times you will be confronted with testing chemicals that you know very little about. I wonder if you have comments about what may be the best way to select the doses for aneuploidy testing?

HSU: Maybe I am making a premature conclusion, but we believe that any agents, such as colchicine, Colcemid, vinblastine, etc., that can cause mitotic arrest, will one way or another induce aneuploidy. Our data have already shown that in each case we got aneuploidy after a period of mitotic arrest. Therefore, for assay purposes, if an agent can arrest mitotic divisions at metaphase, it has the potential to produce aneuploidy. I may be saying this too early, but I think this conclusion will be largely correct. Therefore, there is really no need to count the chromosomes in the recovering populations. All you need to look at is mitotic arrest, using a simple figure like the anaphase/metaphase ratio. But you do need to look at a series of concentrations. We have shown that some agents require very high concentrations, while others require very low concentrations. William Wheeler has been working on the assay of a lot of estrogen and testosterone analogs, and has found that there is quite a variation in terms of ability of blocking mitosis. Some of the analogs are potent; others are less so.

SHARMA: You are putting more faith in the anaphase/metaphase ratio?

HSU: Yes. We use 2 or 3 hr of treatment. If the agent is a mitotic arrestant, the anaphase/metaphase ratio will give us this information. Many people use the mitotic index as a guide. This can also be used, but the results must be compared with the mitotic index of the controls because the mitotic activity varies depending upon the age and initial seeding density of cultures. The anaphase/metaphase ratio gives easier and more reliable data.

SHARMA: Are your anaphase preparation techniques relatively routine or are they still tedious?

HSU: They are routine. You may use the regular harvest methods. We shake out mitotic cells to collect metaphases, so that we have an entire cell population full of metaphases. Then we reverse them and fix the cells at intervals. In one slide we can get about 5 or 6 million anaphases.

SHARMA: Do you have any test in progress of a chemical where you get a differential response in the presence and absence of metabolic activation? Have you done any such studies?

HSU: We have only looked at diazepam (Valium). If you add the S-9 mix, the ability of diazepam to arrest mitosis is reduced.

A MODEL FOR THE MECHANISM OF ANEUPLOIDY INVOLVING CHROMOSOME DISPLACEMENT

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Nondisjunction, the movement of a nondisjoined chromosome (mitosis or meiosis 2) or bivalent (meiosis 1) into one of the two daughter cells, should always result in daughter cells where a chromosomal loss in one is accompanied by a chromosomal gain in another. Consequently, studies showing an excess of hypoploidy have largely been attributed to technical error. Yet most studies, where gains and losses have been recorded, show an excess of hypoploidy and this is often a highly repeatable phenomenon associated with a particular physiological condition such as aging (9). Thus, nondisjunction is insufficient to explain all aneuploidy and a further mechanism leading to frequent chromosome loss must operate in some, if not all, cells.

Ford and Lester (2) described a phenomenon in human lymphocytes which they termed "chromosomal displacement." Displaced chromosomes were those apparently lying in the center of the hollow spindle area when preparations were made without the use of hypotonic solutions or spindle depolymerizing agents, such as colchicine (Fig. 1). Giemsa-banded chromosomes found within the ring were identified and were found to be displaced at a frequency which was primarily dependent on chromosome size (small chromosomes being displaced most often), and to a lesser extent by the amount of C-banded, heterochromatic material in each chromosome. The relationship with chromosomal size was shown to be largely nonchromosome-specific in that a "new" chromosome created by translocation displaced at a rate predictable by its new size rather than by its chromosomal derivation (6).

Chromosome displacement was shown to be related to spindle integrity since the rate of displacement was increased, both with respect to the number of cells showing displaced chromosomes and in the median number of displacements/cell, by unfavorable pH and raised calcium (3,7). More dramatic effects on displacement, accompanied by loss of ring-spindle structure, are seen in response to depolymerizing agents, e.g., Colcemid (8).

Conditions promoting displacement also increase aneuploidy (5) and the relative involvement of the different autosomal human chromosomes in aneuploidy in lymphocytes (i.e., gains plus losses) closely parallels their involvement in displacement (Fig. 2). Thus, it seemed likely that the 2 phenomena could be causally related and a simple model relating displacement and aneuploidy was developed (Fig. 3, Ref. 4). The model relates to mitosis but can easily be extrapolated to meiosis, since spindles will be subject to the same organizational constraints in both processes.

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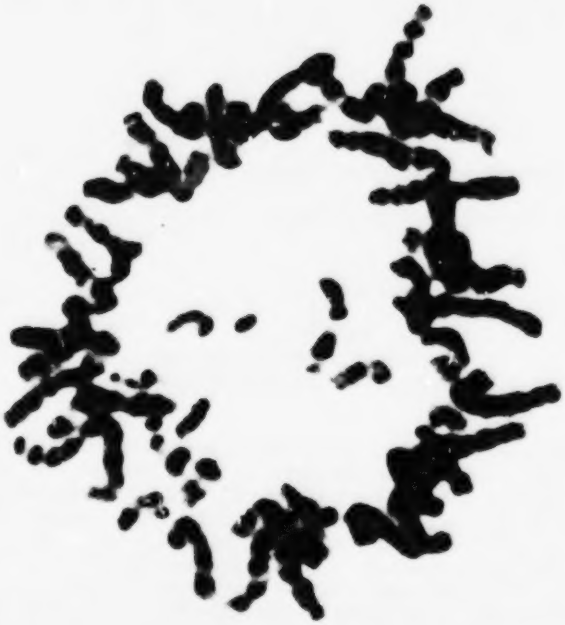

Fig. 1. Metaphase prepared without the use of colchicine or hypotonic prefixation treatment. Displaced chromosomes are those found in the center of the spindle area such that their centromeres are removed from those on the ring by a distance at least equivalent to the length of a chromosome 22.

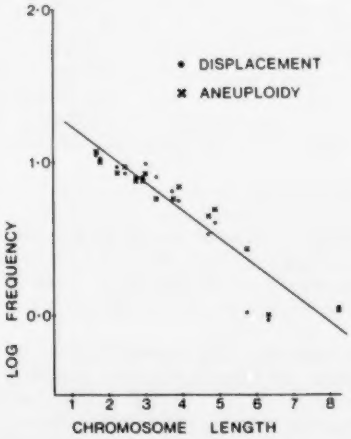

Fig. 2. Graph showing the relationship between chromosome size and the frequencies of displacement and aneuploidy. Frequencies are shown on a logarithmic scale.

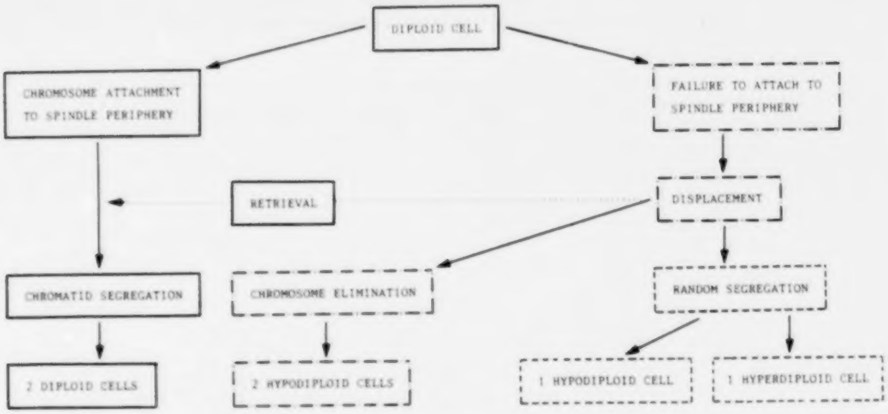

Fig. 3. Model relating displacement and aneuploidy.

The strength of the model may be perceived in its prediction of an alternative mode of chromosome loss which is independent of gain, but which is initiated by the same mechanism. The model predicts that under certain cellular conditions, one or other of the alternative pathways (elimination or random segregation) will be adopted.

In human lymphocytes, elimination would be the more common alternative since loss is usually more frequent than gain. However, under conditions of spindle depolymerization, random segregation would become more frequent, since gains then increase relative to loss (5). Under conditions unfavorable to spindle depolymerization, the ring-spindle structure is gradually lost and with decreasing spindle integrity, displacement approaches 100%. Thus the relationship between chromosome size and involvement in aneuploidy might be expected to reduce concomitantly. Examination of data from human lymphocytes shows that the similarity of the data for chromosome loss and displacement is much greater than that of the data for chromosomal gain and displacement (Tab. 1), although the difference is not significant. Similar changes in involvement of chromosomes in loss and gain have also been found by others (e.g., Ref. 1 and 10). Thus, even this apparent anomaly is predicted by the model and does not detract from it.

The increased X-chromosome aneuploidy seen in older women does not appear to be explained satisfactorily by the model since the X chromosome does not noticeably alter its displacement with age. Thus, an alternative explanation, possibly centromeric dysfunction, is required to explain this phenomenon.

The displacement of autosomal chromosomes into the central area of the hollow spindle has been interpreted as an early step in the misdivision of chromosomes. Displacement is influenced by spindle integrity and is expressed as a function of chromosome size. The model relating displacement to mitotic aneuploidy proposes that displaced chromosomes can be either retrieved or eliminated, or can undergo random segregation. The available experimental data suggest that the involvement of chromosomes in elimination will be more predictable by the equations defining displacement than will their involvement in random segregation. This loss of predictability is a consequence of the spindle disorganization associated with conditions promoting random segregation.

Tab. 1. Involvement of human chromosomes in aneuploidy in PHA-stimulated lymphocyte cultures at 72 hr (pooled results from 16 normal subjects).

Chromosome	Number of losses	Number of gains
1	3	0
2	3	4
3	8	1
4	5	1
5	4	1
6	7	3
7	7	1
8	18	1
9	9	2
10	17	1
11	7	0
12	16	2
13	24	1
14	19	0
15	18	1
16	29	5
17	26	4
18	26	3
19	44	1
20	35	2
21	67	9
22	46	3
Total:	438	46

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DISCUSSION

MAGNI: According to your hypothesis, wouldn't you expect a higher frequency of monosomic cells than of trisomic cells?

FORD: Usually you would. I think you do find that. That has been a problem that people have explained by technical error, even under the most exquisite technical conditions.

[Unidentified Speaker]: I have a comment to make about condensed chromosomes without Colcemid. In most solid tumors, even without Colcemid treatment, we get a very condensed chromosome which cannot band well, and you cannot do much cytogenetic analysis. So we presume that there is some kind of condensing factor in these cells, and present in all cells. But probably in these tumor cells the chromosomes may be much more concentrated, which keeps them very much condensed. So in some individuals in the random human population, there might be a secretion of some substance that keeps the chromosomes condensed much longer.

FORD: If I may make a point. If you take a bone marrow specimen, or a cancer specimen, which comes to you in the laboratory, and you keep it for quite a long time, you might not be able to get division to proceed. But you will have divisions that have actually arrested themselves in metaphase and do not go on. Condensation seems to occur in those cells. So I would say there may be a proportion of cells that just stick in metaphase and do not ever go through anaphase.

ONFELT: When you see these configurations with displaced chromosomes, do you see any other kind of partial C-mitosis? I think this is partial C-mitosis. I find these configurations with different compounds, and usually at very low concentrations. I find at these concentrations three polar spindles, and even more subtle disturbances which I cannot even describe without about 120 photos.

FORD: If you add Colcemid there is a gradual decrease. So if you do a time and dose response of something like Colcemid, you decrease the frequency of those metaphases to zero, with 100% metaphases at the other end. So there is good evidence to show that they are a reflection of the untreated and unColcemidized metaphase--that they are a reflection of spindle orientation when the spindle is not depolymerized.

DISCUSSION: PERSPECTIVES AND FUTURE DIRECTIONS REGARDING THE MECHANISMS OF ANEUPLOIDY

Moderator: B.R. Brinkley

I have been asked by the organizers of this Symposium to offer some "perspectives and future directions" regarding the mechanisms of aneuploidy. Perhaps, in a way, this meeting is historical because for the first time, a number of people from various disciplines have come together, including clinicians who are concerned daily with problems and consequences of aneuploidy, government scientists who must determine the risk of agents producing aneuploidy, counselors who work with patients, and basic researchers who are interested in the fundamental biology and biochemistry of cell division and chromosome distribution. Indeed it is a wonderful opportunity for constructive interaction.

The session today was designed upon the premise that the mechanism responsible for aneuploidy resides in part, if not entirely, with the process of mitosis and the mitotic apparatus. Of course, there are other equally attractive areas where aneuploidy may come into play, i.e., during meiotic prophase when synapsis may result in loss of proper orientation and segregation of homologs, but these topics are dealt with elsewhere in this Volume. There is considerable evidence that a faulty mitosis and an aberration in the mitotic apparatus may be responsible for nondisjunction. In normal mitosis, the fully replicated chromosomes are aligned onto the metaphase plate and are distributed into daughter cells with high fidelity. The frequency of error in yeast is very low (10^{-5}). Although I am not sure whether the error frequency has been calculated for mammalian cells in vitro or in vivo, I am sure that errors are relatively rare. However, after hearing Dr. Nicklas' presentation on the instability of spindle microtubules (MTs) during orientation and reorientation, I am surprised that we ever survived past the zygote stage! Indeed, one is surprised at the numerous, inappropriately arranged kinetochore MTs during prophase orientation and reorientations. But eventually a stable configuration is achieved. Therefore, MT lability appears to be essential for proper chromosome alignment and segregation. It is indeed a seemingly indirect way to build a functional mitotic apparatus but it works very well.

Another thing that impressed me was the apparent redundancy in the mitotic apparatus of higher eukaryotic cells. Yeast, on the other hand, have a small segment of centromeric DNA (250 bp) which is necessary for the interaction with one MT. A few hundred thousand years of evolution have occurred and now cells have greatly enlarged genomes with many chromosomes, large complex centromeres, and numerous MTs attached to a kinetochore

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plate. How redundant is the spindle and to what extent does the redundancy, instability, and capacity to correct an unstable configuration provide a buffer against spindle poisons and environmental toxins that might otherwise induce severe damage and aneuploidy? These are issues which might be worth pursuing in this session.

First, we should review the evidence that an error or damage to the mitotic apparatus can lead to aneuploidy. At this point we should refer to Mazia's definition of a mitotic apparatus: it is a component which can be extracted from mitotic cells and consists of a centrosome at each pole, asteral fibers extending from the poles, kinetochore-to-pole fibers, interpolar fibers, and the kinetochore itself, which anchors the chromosome to the spindle. The chromosomes are not normally included in a definition of the mitotic apparatus because to paraphrase Mazia, they are like a corpse at a funeral. They are the reason for the proceedings but do not take a very active role in the ceremony. We will discuss chromosomes later.

Secondly, based upon the data at hand, I propose that aneuploidy requires cell division. Not everyone may agree with this notion and we can discuss this later. Thus without mitosis, aneuploidy would never be expressed. Other aberrations are possible without mitosis, i.e., endoreduplication, polyploidy, and mutations, but not aneuploidy. It is not possible for a cell to gain or lose chromosomes without mitosis. The evidence for this notion is based largely upon genetics and cytology. In cytological preparation you can see morphological evidence for the loss or gain of one or more chromosomes in the karyotype. In living cells, one can actually see (by video or time-lapse) chromosomes which fail to segregate properly to the pole of the spindle and become excluded from the daughter nuclei. In genetics one can observe evidence of the detection of hemizygosity in the appropriate test.

Finally, many drugs cause aneuploidy and, for the most part, they are spindle poisons which interact with the mitotic apparatus in some way to cause chromosome loss or nondisjunction. At this time, I would like to open this session for discussion. Do you agree with the notion that mitosis must occur before aneuploidy can be expressed?

DISCUSSION

NICKLAS: Well, I understand what you are saying. It is not going to be revealed unless you have cell division. But I am sure you would agree that you could have errors in DNA replication that would result in aneuploidy.

BRINKLEY: Let's expand on that a bit. Do you mean unscheduled DNA synthesis or endoreduplication could lead to loss or gain of chromosomes?

NICKLAS: Don't you imagine that you could have a failure to replicate a chromosome and lose it that way?

BOND: May I make a contribution by giving you a quote from Charles Ford who wrote, "Is there an alternative [to nondisjunction]? Let us take trisomy first. The only possibility appears to be the generation of an extra chromosome of specific type by two periods of DNA replication in that particular chromosome, while the remainder go through one." I do not dispute that aneuploidy arises through nondisjunction, but I think the word "requirement" is logically untenable. There are many examples in the literature where you could argue that DNA replication has generated aneuploidy.

NICKLAS: To me, the more interesting point is that I know of one good evolutionary example where you get what is "normal" aneuploidy (if you can tolerate such a term), which does not require mitosis. In Sciara, there are losses of chromosomes that are associated with setting up the "proper" number for the soma. Now those losses for the somatic cells are cell division-associated; they involve failure of anaphase movement. But in the germline, they start off with three X chromosomes; males lose two of these chromosomes in the germline, females lose one. They lose these chromosomes (it is very well established) by the migration of the chromosome through the nuclear envelope during interphase. So evolutionarily, there has been a most amusing, and a still, unfortunately, insufficiently studied example where you can lose a chromosome specifically (you probably wouldn't call it aneuploidy; I probably shouldn't either) during interphase.

BRINKLEY: Well, in my view it is a manifestation of aneuploidy. Are there other comments? Can there be partial endoreduplication?

HSU: I might comment about this so-called partial endoreduplication business. Many of you who are not cytogeneticists may have heard this term coined by Lejeune years ago. In a syndrome of children, and parents too, he found that in one of the chromosome 2s in metaphase, the long arm had reduplicated and the short arm had not. All the rest of them were normal. He called this partial endoreduplication. As it turns out, this was not the case. What happened was there was a chromatid break in the previous division, a fragile site, right near the centromere. During the division (anaphase), a chromosome (naturally the sister is normal) and a sister had a break. The broken fragment went with the sister to the daughter cell, and they then both replicated. The normal chromosome and the broken fragment replicated. That is why you get this kind of morphology. Therefore, it is not differential endoreduplication, it is just a fragment.

MCINTOSH: As I understand it, a single chromosome is rarely a single replicon. Is there some kind of control that would allow all those replicons on one chromosome to fire such that you could duplicate that object, rather than simply one individual replicon? Or, if not, are there abnormalities where one becomes aneuploid for a set of loci which are on a chromosome, but not for the whole chromosome?

BRINKLEY: I do not know of any. Dr. Moens?

MOENS: This is the work from Shimky, I think. He arrests the cells in early S-phase and then releases them. Then S-phase starts all over again, and all these regions can be incorporated with recombination. So you get duplicated regions of a number of early genes.

BLOOM: Dr. Resnick reminded me of this work out of my laboratory. I'm just beginning to think more about aneuploidy. This is a great meeting for me. In the construction that we have made where we can turn the centromere on and off, we have a situation in which, under conditions where the centromere is working, it is replicating once per cell division and being segregated. When we now switch the carbon source, that is, in effect, aneuploidy. Now it continues to replicate and you get an increased number per cell without necessarily cell division. So it is an artificial thing that we have constructed. But if we were able to construct it, I am sure it happens in cells.

BRINKLEY: Perhaps we can generally agree that for aneuploidy to be expressed, you must have cell division. We should now consider more specific targets.

From what we heard Dr. Bloom say about yeast, perhaps we can begin to ask the question, does a chromosome have encoded in its centromeric sequences some kind of information that will determine not only its mode of transportation, but also its direction of movement? From an earlier discussion we learned that if centromeric DNA sequences are deleted, you get no chromosome movement or segregation. This small piece of DNA which resides in the yeast centromere contains information that leads to coupling of the chromosome with the mitotic apparatus in mitosis, and to directed movement, equational separation, and alignment of the chromosomes to facilitate reductional division in meiosis. Obviously, more molecular biology studies of the centromere in eukaryotic organisms are needed. For example, it should be possible to clone centromeric DNA sequences from chromosomes of other eukaryotic organisms, including mammals. The technology for achieving this is available, but there are many problems. For example, the DNA in the centromere of a human chromosome is many orders of magnitude greater in size and complexity than the DNA in the centromere of the yeast. Moreover, the important sequences may be small and relatively insignificant when compared to the abundant repetitious DNA in the centromere of mammals. From a simplistic logic, we might conclude that the centromeric sequences in mammals would be multimers of that in yeast. This conclusion is based upon the fact that yeast have one MT per centromere, while man has about 18 MTs per centromere. Is it therefore possible that some aneuploidy-producing drugs bind directly to centromeric DNA to alter chromosome segregation?

RESNICK: I think it is a great idea. Dr. Bloom and I are, in fact, trying to pursue this very issue through our labs' collaboration. We are specifically looking at the issue of DNA damage in the specific centromere region that is important for chromosome segregation. And so far the only probe that we have used is UV radiation, just because it is convenient and very cheap. You also know the types of damage it produces. What we have found is that in the essential region of the centromere is a hot spot. (In fact, there appears to be two hot spots.) Now maybe this could explain an UV-induced aneuploidy. I am not really sure. We are trying to investigate that issue. This also may be rather fortuitous because the essential region (I think it was region 2 on Dr. Bloom's map) is about 80 or 90% AT-rich, so it is not very surprising. But we would like to adapt these techniques to some other types of damage, say that induced by alkylating agents, and see whether, in fact, they might disrupt the association between the centromere and the centromere protein, which is what Dr. Bloom has been looking at.

MAGNI: Fortunately, we have some clear evidence against this hypothesis by Dr. Resnick. We use a system where we study nondisjunction in meiosis in yeast. We tested many compounds which more or less induce nondisjunction, but the 5 DNA ligands so far tested, such as acridines, are absolutely negative in this respect. Other antibiotics specific for making ligands with DNA are the only compounds which are completely negative in inducing nondisjunction. We do not know why but it is experimental evidence.

BRINKLEY: There are alkylating agents that cause aneuploidy in test systems, but we do not yet understand the mechanism of their action.

WARBURTON: Does anybody have any idea how many centromeric probes we are going to have to find in order to study all the species that we want to study? Because as far as I know, there is not any homology between the yeast centromeric probe and mammalian chromosomes. Do the people who have such a probe know what it does homologize with? Is there any homology with anything else, or are we going to have to find a different probe for every mammal? How big a task do we have ahead of us?

BLOOM: In fact, the experiment is just being done. First of all, if you take the piece of yeast DNA that has centromere function, Dr. Warburton is right, in that it does not cross-hybridize with any other yeast chromosomes, much less any other chromosome in any other organism. What we are just being able to do now with the advent of oligonucleotide synthesis is to synthesize the small region of homology that is conserved in the yeast centromere (these elements 1 and 3 that are 14 bp and 11 bp, respectively). The experiment would be to use these elements as hybridization probes to higher organisms. This may be the much more critical experiment for use as a hybridization probe. The one problem with this experiment that I anticipate is that 14 and 11 bp are so small, that for a human genome, they will certainly light something up, so it is not clear whether you can make a definitive statement and say, if they do light something up, that that is the centromere. You might have to make a consensus oligonucleotide that would be 14 bp, or that would be ~19 bp. Then if that lit something up, you could say with more confidence that it is a real centromere. But I think using the oligonucleotide probes is going to be the way to go in that respect.

BRINKLEY: Then it does seem logical to consider for future studies the possibility that centromeric DNA sequences are important, and we should look for ways to test them. Let's move on from the chromosome to another structure, and that is the mitotic apparatus itself. We heard quite a bit about this earlier in the Symposium. There have been a lot of new things learned lately about the mitotic apparatus. For many years we looked at it in the electron microscope with fascination. Through the use of polarity markers as discussed by Dr. McIntosh, we are beginning to understand the polarity of spindle fibers. Can we make any predictions on how chromosome movement takes place? Are MTs important in chromosome movement? Clearly they are, because you can disrupt them with colchicine and chromosome movement stops. But are MTs the force producing organelles--the motors? Dr. Nicklas mentioned that the motor was at the poles. It seems logical therefore that a faulty motor could lead to arrest of chromosome movement or nondisjunction. Force production per se and the identification of force-producing molecules responsible for chromosome movement are things we should consider in greater detail. Something drives the chromosome, and something produces a force. It is amazing that we know so much about force production in muscle, cilia, and flagella, and yet we still have very little knowledge of the forces that move chromosomes. Dr. Nicklas has reported that it takes only a very small force to move a chromosome.

RESNICK: Do you expect that there is a force that actually pulls the centromeres apart in rows that move?

BRINKLEY: Again, Dr. Nicklas is the expert. He has reported that the force works through the kinetochore toward the poles.

NICKLAS: Are you talking about whether there is a force that drives the poles apart?

RESNICK: There is a force involved in moving these chromosomes to the poles. What is the force that pulls them apart?

BRINKLEY: Most of the evidence suggests that chromatid separation occurs independent of spindle function in most species. That is, if you block with colchicine long enough, the chromatids fall apart independent of MTs.

DE BRABANDER: I think there is pretty good evidence in plants, and a number of people have been trying to find the same evidence in mammalian cells (in human lymphocytes, etc.). Sometimes when making chromosome spreads, when there is little separation of the centromere region, this is taken as evidence for chromatid separation in cells blocked with Colcemid, etc. But it is not true evidence. We looked for it in many different ways, and we could not find any evidence whatsoever. In fact, we found evidence to the contrary. One set of experiments which we did was similar to what Dr. Hsu has been talking about. If you treat cells with colchicine or nocodazole or another specific MT inhibitor, you can arrest them for about 6 hr; they then begin forming restitution nuclei, etc. Now this process is followed with different kinds of techniques. We have been using kinetochore staining with the human autobodies and, recently, high resolution video contrast microscopy. All the chromosomes that we followed with the video microscopy did not separate before the cell went into telophase. With the kinetochore antibodies too, we could not find any evidence of separation. So in the restitution nuclei, at the early phase, you always find two kinetochores close together, also showing that there is no real separation. There is a third set of evidence in PTK cells (they each have one very nice nucleolus). If you block them, and you release them after a treatment time longer than about 1-2 hr, then again, before that time, there is formation of a spindle, chromatid separation, normal anaphase, etc. If you block them for more than 2 hr, there is nucleation of MTs on the centrosomes and the kinetochores. There is formation of a very normal prometaphase-like spindle. Then all of a sudden you get telophase before anaphase has occurred. So apparently if the chromosomes are rested too long, they will not separate even if you let them make a normal spindle. There is a deregulation in the timing of telophase. What you see in these situations is that, invariably, one of the daughter cells has a big nucleolus, and the other daughter cell has no nucleolus, which means that this pair of chromatids always goes to one of the daughter cells.

NICKLAS: Would you therefore argue what I would regard as almost a corollary to such a view, that normally the spindle, in fact, pulls these things apart willy-nilly? That is incredibly doubtful to me.

DE BRABANDER: No, no. I am not implying that.

NICKLAS: There are two different questions here. The first question is: can you get evidence for morphological separation after you poison the spindle? That is what Dr. De Brabander is talking about. The second question is: are the chromosomes, in fact, pulled apart willy-nilly by spindle forces? I say the answer to that is plain: no! That is not true. There is a separate step in which they fall apart. You can document this very directly by trying to separate chromosomes in meiosis with a micromanipulation needle.

BRINKLEY: Dr. Gaulden has an interesting point to make in this regard.

GAULDEN: I would like to call the attention of everybody to this new protein that has been discovered recently: the P1 protein on perichromatin. This may be one of the things that is holding the chromatids together on the metaphase spindle. In the grasshopper neuroblast in the living state, at the minute the centromeres begin to separate, the chromatids repel each other. This is an act independent of the centromere. If you have an acentric fragment produced, the parts of this fragment will separate at the same time as the centric fragments do. So this seems to be independent of it. We have evidence that indicates that there are some chemicals, such as formaldehyde, that will prevent the separation of the centromeres. I think we also get this with mitomycin C. The centromeres try to go to the poles, but they cannot separate.

MCINTOSH: I think that there is an aspect of spindle function which none of us has yet dwelled on, which is worth mentioning in this context. Spindle action is controlled in ways that are only very dimly understood. One aspect of the control is a membranous system that permeates the spindle of a lot of higher plants and animals. This membranous system has been isolated along with spindles by means of using an isolation that does not use detergents, and it seems to be able to concentrate calcium ions. There is some evidence that there is a calcium ion release that occurs sometime around the metaphase-to-anaphase transition. This comes from fluorescent studies in Peter Hepler's laboratory, using probes that bind to membranes, like chlorotetracycline, and change their fluorescence as a function of change in calcium ion concentration. Then there are other experiments in which, if you inject calcium ions into a metaphase cell, they seem to induce a precocious anaphase. What I want to point out is that if that system is functioning normally, it probably gives rise to a humoral control which is essentially universal throughout the spindle. And yet you could imagine a pore type of function of the kind which could give rise to precocious anaphase in one place or laggard anaphase in another, and that this control system may possibly be another site for an aneuploidy-inducing function.

ÖNFELT: I would like to comment on membranes. I am reminded of the very old data from the 1940s by Estergavin and Lavanne and several others showing that many compounds can induce spindle disturbances as observed indirectly through C-mitotic events. They found that the threshold for this effect, with all these compounds, shows a relationship with the lipophilic character of the compounds, or rather, in those studies, water solubility. They found that the higher the water solubility of the compound, the higher the concentration that was required to achieve C-mitosis. Some of these old data have been reevaluated by Hunch and Dunn, who, instead of using water solubility, expressed lipophilic character as the partition coefficient in octanol water. They found that C-mitosis was one of the biological endpoints which were the most sensitive to hydrophobic forces. You could argue then that many compounds will induce spindle disturbances. They will induce them by combining, or attaching to, hydrophobic compartments that could be membranes, or that could be hydrophobic pockets of proteins. There are really many possibilities. Furthermore, I have studied about 20 different compounds in V79 Chinese hamster cells, and have found that all but one of these compounds induce an increased frequency of aneuploidy in these cells. So I think that we should consider the possibility that the lipophilic character of the compound, and that membranes and hydrophobic pockets of proteins, are very important targets. One of the compounds I have investigated induces increased lipid peroxidation. When I pretreat cells with very low levels of vitamin E, or sodium celanate, this pretreatment has a sudden protective effect. The frequency of C-mitosis is lowered with this compound. Then I find that, for instance, the compound which is the substrate for the very important protective enzyme, glutathym-peroxidase, which is selenium-dependent, is tertiary butylhydrylperoxide. This compound induces C-mitotic events at very low concentrations. The dose response, or the concentration response, for C-mitosis is very peculiar, which I think might suggest that this enzyme has an important role during mitosis. It may be involved in the turnover of glutathione during mitosis, which has been suggested from studies on sea urchin eggs and, I think, from the increased activity of glutathione reductase during mitosis. To conclude, membranes may be a very important target.

BRINKLEY: Thank you for bringing up membranes, because I think we were remiss in not mentioning them. In fact, membranes are very important components of the mitotic apparatus. Smooth-surfaced membrane vesicles extend along the spindle fibers from pole to pole, which are thought to contain calcium. The calcium-binding protein, calmodulin, extends along the kinetochore-to-pole fibers in the half spindle, and are juxtaposed to the membranes. This has led some to propose that calmodulin may be the intermediate in transport of calcium ions in the stored smooth-surfaced membranes to the mitotic spindle where they disrupt the MTs.

MOLECULAR BIOLOGY OF MEIOSIS: SYNAPSIS-ASSOCIATED PHENOMENA

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INTRODUCTION

In tracing the metabolism of nuclear DNA during meiosis we have identified two groups of DNA sequences, each displaying a distinctive pattern of behavior during meiotic prophase. One group, which will be referred to as zygotene DNA ("zygDNA"), [illegible] coordinated in most of its activities with chromosome pairing. The other group, referred to as pachytene DNA ("PDNA"), is coordinated in its behavior with what we believe to be the process of crossing-over. Each group has been and continues to be identified by its respective mode of replication during zygotene and pachytene, well after the apparent completion of the premeiotic S-phase. The theme of this chapter is that chromosomes of at least the larger eukaryotic genomes have evolved certain DNA sequences that are specifically addressed to particular chormosomal needs during meiosis. In this chapter we focus on the zygDNA sequences because of their apparent relationship to chromosome pairing. We have no direct evidence that this is indeed the case, but we do know that the behavior of zygDNA during meiotic prophase provides strong evidence for its playing an important role in the process. Our description of that role is necessarily conjectural and our interpretation of various findings are slanted in favor of the conjecture.

PROPERTIES OF zygDNA

General Characteristics

ZygDNA comprises a group of DNA segments measuring as much as 10 kbp in length and constituting, by recent estimates, about 0.1-0.2% of the *Lilium* genome. Depending on conditions of preparation, purified segments range in size from 2.5-10 kbp (5). The actual length of the segments and whether or not they are uniform in length remain uncertain. When subjected to C_ot analysis, most of the zygDNA behaves like unique or low copy number sequences and thus contrasts with the remainder of the genome which consists largely of a wide range of repeated sequences (2). The segments of zygDNA are widely distributed among all the chromosomes, a distribution that is demonstrable by autoradiography (Fig. 1). In early studies it was shown that partial inhibition of zygDNA synthesis resulted in fragmentation of all chromosomes (6).

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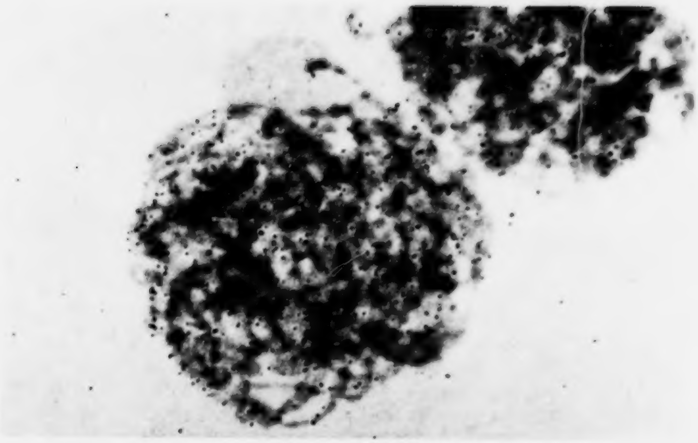

Fig. 1. Autoradiograph of Lilium pachytene nuclei previously hybridized with 3H zygDNA. Hybridization was performed in presence of 20 x unlabeled nuclear DNA to reduce the effects of impurities in the zygDNA preparation. The broad distribution of grains is evident in the photograph. Similar results were obtained when metaphase 1 chromosomes were the hybridization targets. (Y. Hotta, unpubl. data)

Distinguishing Characteristics

ZygDNA may be selectively labeled by pulsing microsporocytes with [^{3}H]-thymidine during the zygotene stage. DNA thus labeled can be separated as a high density fraction when total nuclear DNA is centrifuged to equilibrium in a gradient of CsCl (12). The labeling is due to a semiconservative replication of zygDNA that was suppressed in its replication during the premeiotic S-phase and during subsequent stages prior to zygotene. The occurrence of a semiconservative replication after completion of the premeiotic S-phase is unique to zygDNA and, to our knowledge, the existence of 2 and possibly 3 separate intervals of DNA replication following the S-phase is a unique feature of meiotic DNA metabolism. The temporal correlation of zygDNA replication with chromosome pairing is strongly suggestive of a functional relationship between the two processes.

The distinctiveness of zygDNA behavior is not only reflected in its replication during zygotene, but also in the attenuated pattern of that replication. Although zygDNA is largely replicated during zygotene, small flanking regions, and possibly interstitial ones, within each of the zygDNA segments remain unreplicated until about the time of chromosome disjunction (Fig. 2). It is this unusual behavior that has made it possible to identify zygotene DNA. Single-strand gaps are susceptible to breakage either by S1 nuclease or by controlled mechanical shearing. Both procedures have been used to excise zygDNA segments from pachytene meiocytes (Fig. 3). Although zygDNA constitutes a very small fraction of the Lilium genome, its pattern of replication during meiotic prophase points to its having a structural role in the meiotic process. There is also some evidence, though limited, that the mouse genome has a DNA component similar in its meiotic behavior to lily zygDNA (5). If the evidence for this could be

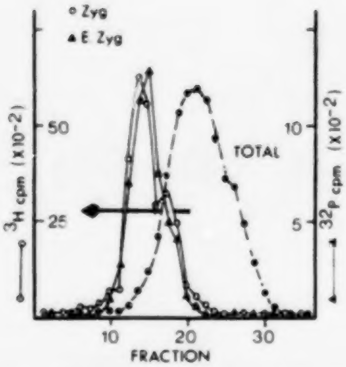

Fig. 2. Releasability of zygDNA from meiotic nuclei. Profiles of the DNA samples were obtained by centrifuging the preparations to equilibrium in CsCl gradients. Direction of increasing density is shown by the arrow. The "zyg" profile is of DNA released by treating isolated nuclei from early pachytene cells with S1 nuclease and purifying the DNA from the released material. The cells had been incubated in the presence of [^{3}H]-thymidine during zygotene and harvested in early pachytene. The label occupies the region of the gradient typical for zygDNA. The "E.Zyg" profile is of DNA similarly prepared but from nuclei isolated from late leptotene-early zygotene cells. In this case, the DNA was end-labeled in vitro with [^{32}P] ATP so that the cells were not incubated prior to nuclear isolation. The virtual coincidence of the two profiles is clear as is the difference of their mean buoyant densities from that of "Total" nuclear DNA. Leptotene and preleptotene nuclei treated in this way do not release zygDNA unless the nuclei are first extracted with deoxycholate as discussed in the text. (Y. Hotta, unpubl. data)

more fully established it would add considerably to the viewpoint that zygDNA is a widespread chromosomal component with at least one specific meiotic function.

Functional Properties

As already mentioned, the temporal coordination of zygDNA replication with chromosome synapsis in itself suggests the existence of a functional relationship. This possibility is given added weight by 2 other observations. Roth and Ito (10) demonstrated that interference with DNA replication during zygotene interfered with chromosome pairing and synaptonemal complex formation. Kurata and Ito (7), using electron microscope autoradiography, found that microsporocytes pulse-labeled with [^{3}H]-thymidine during zygotene displayed a concentration of DNA label in the region of the synaptonemal complex. A similar observation was made in mouse meiocytes by Moses et al. (8). There is thus fairly strong circumstantial evidence that zygDNA replication is linked to the process of chromosome pairing.

Inasmuch as the suppression of zygDNA replication during and immediately following S-phase is unique to meiosis, it must be linked to a regulatory mechanism that is also unique to meiosis. The existence of such a mechanism has now been established and it represents the first identified molecular event that precedes meiosis and is essential to its development (5). Once that suppression has occurred, the different treatments that

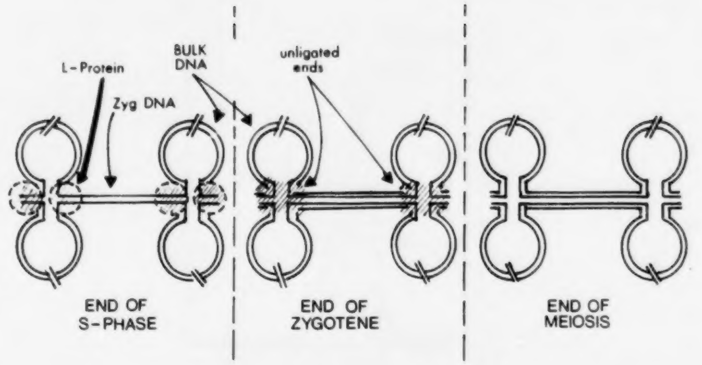

Fig. 3. Diagrammatic representation of zygDNA behavior during meiosis. A chromosome region housing one zygDNA segment is used for illustration. The zygDNA is represented as though constituting part or all of a chromosome axis, more than 99% of chromosomal DNA being subtended from the zygotene regions. The absence of zygDNA replication at the end of premeiotic S-phase is shown, as is the presence of L-protein bound to the ends of the zygDNA segments. Although replication occurs at zygotene, the ends of the newly replicated strands are separated by small gaps from the bulk DNA strands, thus making possible excision by S1 nuclease as discussed in the text. At the end of meiosis ligation is complete and zygDNA can no longer be separated as a pseudo-satellite in a CsCl gradient.

have been used to cause premeiotic cells to revert to mitosis are no longer effective. Suppression of zygDNA replication coincides with an irreversible commitment of meiocytes to enter meiosis. As expected, if meiocytes are induced to enter mitosis, they complete zygDNA replication before doing so. It is significant that physiological perturbations of the meiocytes following commitment, but prior to the onset of leptotene, result in much-reduced chiasma frequencies (9). It would appear that the premeiotic interval beginning at about the termination of S-phase includes events essential to the occurrence of normal synapsis. These events are obviously relevant to the subsequent occurrence of aneuploidy in gametes.

THE L-PROTEIN

The factor inhibiting zygDNA replication is a protein having a molecular weight of about 73,000 daltons and has been called the "leptotene" or "L"-protein (4). It is most probably synthesized toward the end of the premeiotic S-phase, although its presence earlier in an inactive or unextractable form cannot be excluded. It is desirable to describe the procedures used in identifying and characterizing the protein because the evidence for its function is based entirely on a series of in vitro experiments. These experiments were feasible because of earlier observations that DNA behavior in isolated nuclei mimics its behavior in situ, at least with respect to replication. Thus, if isolated nuclei from a particular meiotic stage are incubated with deoxynucleotide triphosphates, the pattern of replication that is observed is the same as the one that occurs in vivo. Nuclei isolated from zygotene cells replicate zygDNA, whereas those from leptotene cells display very little replication. Addition of DNA polymerase to the medium increases the rate of replication but does not alter the pattern. Post S-phase but prezygotene nuclei lack replicated zygDNA segments, and although nuclei from earlier or later developmental stages can synthesize DNA in vitro, those prepared from the interval between the two stages do not. The availability of zygDNA probes made it possible to address the question of why zygDNA displayed such distinctive behavior between premeiotic S-phase and zygotene.

The first indication that chromatin structure in zygDNA regions during leptotene might differ from that during zygotene was the finding that the unreplicated zygDNA could not be excised from the chromatin mass by S1 nuclease as was the case when zygotene nuclei were so treated (Fig. 3). This observation made it unlikely that the segments of zygDNA were present as single strands following S-phase, although the possibility could not be excluded that associated proteins protected the DNA against S1 digestion. The implication of the finding itself is not trivial because a persistence of zygDNA in duplex form following S-phase requires that sister chromatids be tightly apposed, each member of the duplex being contributed by one of the chromatids. Isolated nuclei from any of the prezygotene stages that are treated under the same conditions as zygotene nuclei do not release zygDNA. If, however, prezygotene nuclei are briefly treated with deoxycholate (DOC), a treatment that removes various components of the nuclear membrane, incubation of the nuclei with S1 nuclease now releases zygDNA segments, and they are released as duplex fragments. The effect is not observed if treatments are carried out with various nonionic detergents even though such treatments extract a number of nuclear components. The effectiveness of deoxycholate, coupled with the ineffectiveness of the nonionic detergents, has made it possible to purify the factor involved. The nature of that factor aside, the observations on the prezygotene behavior of zygDNA make it highly likely that sister chromatids are tightly joined prior to the initiation of chromosome pairing.

The pointer to regulation of zygDNA replication was provided by observations that DOC not only rendered zygDNA releasable by S1 nuclease treatment of isolated nuclei, but also made it possible for the nuclei to replicate zygDNA when incubated under appropriate conditions. The deoxycholate was found not to be a general stimulant of DNA synthesis in isolated nuclei. Premeiotic cells in S-phase and postmeiotic microspores in S-phase showed little effect; nuclei from somatic cells were also unresponsive. Clearly, in prezygotene cells a DOC-extractable factor is present that, if removed, permits the replication of zygDNA during prezygotene stages. The effect on the replication of other DNA sequences is negligible.

Using simple methods of purification, the factor responsible can be identified as a protein ("L-protein"). DOC-treated leptotene nuclei can be suppressed in their newly acquired capacity to replicate zygDNA if exposed to L-protein or to an extract containing that protein. Such active extracts are obtainable from nuclei spanning a short interval of meiotic development. Nuclei from midzygotene and later stages, and so too those from somatic cells, are poor sources of the protein. Indeed, it was somewhat surprising to find that the premeiotic S-phase nuclei had little L-protein in their DOC extracts. The time at which L-protein appears in the extracts coincides roughly with the time at which the meiocytes become irreversibly committed to meiosis. Determining the precise sequence of these two events is beyond the reach of our experimental procedures. It is nevertheless clear from the analysis of DOC extracts prepared from cells at successive developmental stages starting at premeiosis that L-protein is absent during most of the S-phase and must therefore be formed near or at the end of S-phase. The absence of zygDNA replication during the S-phase could be due to the presence of a different meiotic factor that effects the suppression. Or, zygDNA sequences may be normally programmed to replicate at the end of S-phase in all cell cycles, so that the formation of L-protein for meiotic purposes is not needed until that time. Such an explanation implies a special function of zygDNA in mitotic divisions, a function that could be speculatively assigned to a regulation of replication of the hypothetical chromosomal axis. Replication of zygDNA would thus determine the time at which sister chromatids are fully formed, a situation that is not achieved until well after S-phase in meiotic cells.

It is to be expected that the specific suppression of zygDNA replication by L-protein would be matched by a corresponding specificity in binding between L-protein and zygDNA. This has been tested and shown to be the case. Purified zygDNA binds L-protein, whereas the remainder of the genome (99.8% or more of the total) does not. Because zygDNA consists mainly of single or low copy number sequences the general binding of the L-protein is unlikely to be due to a specific affinity of zygDNA sequences as a whole for the protein. Experiments with some individual clones of zygDNA sequences make it possible to determine the fraction of the cloned segment that is involved in binding, and also the extent to which other DNA types compete with the zygDNA for binding to the L-protein. When segments of zygDNA, 2,600 bp long, are bound to L-protein and then treated under controlled conditions with DNase I, protection against digestion is provided for about 90 bp (4). How much of the sequence is directly bound to the L-protein is not revealed by these measurements, but it is evident that a very small fraction of the zygDNA sequences is involved in the binding reaction. The cloned fragment of zygDNA does not include the entire zygotene segment and, depending on the actual size of the segment in situ, the cloned fragment may represent no more than 25% (assuming a value of 10 kbp). If only one binding site were present in each segment all the L-protein binding sites would involve no more than about 1% of zygDNA and thus about 0.002% or less of the entire nuclear genome. The value, of course, is a rough one because of the many approximations made in estimating the actual size of zygDNA segments, the number of binding sites per segment, the size of the binding site, and the assumption of 100% purity in the zygDNA preparations. It also remains to be determined whether one or several sequence types bind to L-protein. Nevertheless, it is reasonable to draw the tentative conclusion that the complex array of zygDNA sequences contains a subfamily of sequences that has a specific affinity for L-protein.

One unanticipated property of the L-protein that was discovered in the course of examining its binding characteristics is almost certainly significant to the meiotic process. Meiosis-specific, DNA-binding proteins, when such are discovered, are always tested for topoisomerase activity primarily because proteins capable of effecting topological changes in DNA are potentially important as mechanisms in both pairing and recombination. To do so, we exposed supercoiled plasmids to the L-protein. Plasmids containing zygDNA with and without L-protein binding sites were tested, and so too were plasmids without zygDNA inserts. No effect was detected on plasmids that did not bind L-protein regardless of whether the plasmid did or did not contain zygDNA sequences. However, those zygDNA plasmids containing the binding site relaxed from their supercoiled condition if ATP was present in the medium. The relaxation was not due to topoisomerase activity. L-protein has a very different action when bound to duplex zygDNA; it introduces a single nick into only one of the strands. When plasmids so affected are denatured the products are a single strand circle and a single piece of linear DNA. Presumably, the ability of L-protein to introduce one nick at a particular site in vitro reflects a corresponding action in vivo. That property is distinctive enough to point to a special meiotic function, but apart from the belief that the function is related to the pairing process, specific mechanisms of action cannot be inferred from the observations that have thus far been made. It may be of interest to note that positively supercoiled plasmids housing the L-protein binding site do not bind L-protein and, therefore, do not nick the plasmid.

The fact that L-protein has the capacity to: bind selectively to specific regions in zygDNA, selectively suppress zygDNA replication, and introduce a site-selective nick in coordination with binding points to its having more than one meiotic function. The nature of the functions is subject to speculation. Circumstantial evidence points to those functions being addressed to chromosome pairing and, in the absence of any pointers to the contrary, we presume this to be the case. Suppressive action of L-protein may be conveniently accounted for by supposing that a precondition for chromosome alignment is the presence of unreplicated zygDNA in an otherwise replicated chromosome. This precondition needs to be linked to another requirement because pairing could occur near the end of S-phase when the rest of the genome, but not zygDNA, has undergone replication. We suppose that pairing does not occur then because zygDNA functions only in aligning homologs and alignment requires the presence of a stabilizing mechanism to be effective. The components of the stabilizing mechanism (synaptonemal complex) must be functional at the time of alignment so that the signal to provide those components must occur very close to the time at which zygDNA replication is suppressed by the L-protein. That is the time when microsporocytes become irreversibly committed to meiosis (9).

zygDNA ACTIVITY IN SYNAPSIS

The nature of the role that zygDNA might play in chromosome alignment is open to wide speculation. If there were evidence to indicate that the zygDNA segments constituted a chromosomal axis or a critical part of such an axis, a model in which these axial elements furnished the sites for homologous matching would have a sound basis. The localization of L-protein in the nuclear membrane or a related lipoprotein component could be taken to indicate that the process of alignment occurs with a membrane structure, a condition that, because of its 2- rather than 3-dimensional nature, would enhance the probability of encounters between homologous regions. Coordinating the initiation of replication in a particular zygDNA segment with the initiation of homologus synapsis may well facilitate the process because replication of the DNA duplex requires a separation of its complementary strands. Such separation could transiently provide single strands in each of the homologous zygDNA segments, thus allowing for cross-hybridization between complementary DNA strands. There is, moreover, a temporal factor that would facilitate synapsis in this system. ZygDNA segments are all coordinated but are not simultaneous in their replication. Relatively few must replicate at any one time during zygotene. If it were assumed that a regulatory mechanism exists for a simultaneous initiation of replication in homologus segments, this temporal factor would significantly reduce the number of DNA segments available for matching at any given time. The reduction in complexity of the hybridizing pool of segments coupled with the 2-dimensional area in which hybridization occurs would significantly increase the efficiency of matching. The one critical question that remains is whether any of these postulated relationships exist.

The possible role of L-protein's nicking activity may be considered. An activity that seems to be tightly linked to a specific group of DNA sequences must surely function in coordination with the activities of those sequences. From the standpoint of matching and heteroduplex formation, the introduction of a nick into one strand of the unreplicated zygDNA segment would make possible the formation of a single-strand tail that could interact with its homolog. Once the alignment had been stabilized replication could proceed without disturbing synapsis. There are, of course, other functions that can be directly related to zygDNA behavior, some of which might indirectly involve the nicking action in synapsis. The nicking could be a signal for initiating zygDNA replication. And, as discussed below, it could be involved in the initiation of zygDNA transcription.

In closing this aspect of the chapter, it is useful to summarize the principal points made concerning the role of zygDNA in the pairing process. We assume that the homologous alignment of chromosomes is a process distinct from one that stabilizes alignment. When stabilization is coordinated with homologous alignment, homologous synapsis is the result. When not so coordinated, stabilization of heterologous alignment may be the result. We speculate that zygDNA functions only in alignment. Its delayed replication, however, is a requirement for alignment but does not initiate the process. Other, as yet undetermined, factors must be involved in the initiation of meiotic pairing. ZygDNA may be viewed as an axial component of each chromosome that functions to align it with its homolog after partial compaction has occurred. The meiosis-specific L-protein regulates the timing of zygDNA replication and is also active during the process of alignment. The nicking action of L-protein provides a source of single-strand tails. Their formation and the subsequent formation of duplexes between complementary strands derived from homologous chromatids are activities which can be secured by the meiosis-specific "U-protein" and "R-protein," components that are formed either prior to or during early zygotene (11). These proteins, which can be fitted into schemes for synapsis and recombination, are present only during zygotene and pachytene. They may function in one or both of these fundamental meiotic processes.

zygDNA: TRANSCRIPTION

It has been recently discovered that zygDNA not only behaves as a structural component in the synaptic process, but that it also is a source of poly(A)RNA transcripts during the interval of chromosome synapsis (5). If extracts are made of poly(A)RNA at different stages of meiotic prophase a significant fraction of the poly(A)RNA population hybridizes with zygDNA. Two features of this hybridization point to its being highly relevant to meiosis. Similar extracts from mitotically proliferating tissue lack significant amounts of what may be termed "zygRNA" sequences. The transcripts appear to be exclusively, or nearly so, products of meiotic activity. Poly(A)RNA extracts of neither premeiotic cells nor postmeiotic microspores contain appreciable amounts of zygDNA hybridizable transcripts. Within the meiocytes themselves the cycle of zygRNA formation and disappearance is sharply defined such that the window of its presence spans a very narrow interval of meiotic prophase, extending from late leptotene, peaking at early zygotene, and virtually disappearing by midpachytene. It would appear that zygRNA is a product whose formation is required at the early stages of synapsis. The importance of zygRNA to meiosis is suggested by the fact that at its peak concentration it accounts for 40% of the total poly(A)RNA population. Moreover, the poly(A)RNA population of prophase meiocytes from the achiasmatic *Lilium* hybrid has little zygRNA. Whether the low level of zygRNA is cause or consequence of pairing failure is yet to be established, but it is at least apparent that there is a fairly close relation between homologous synapsis and the presence of zygRNA transcripts.

The coordinated production of a group of meiosis-specific poly(A)RNA transcripts with chromosome pairing is in itself of major interest, both because of the apparent uniqueness of zygRNA to meiosis and also because of its temporal correlation with synapsis. However, it acquires even more interest when it is considered that the DNA sequences from which zygRNA is transcribed are part of a genomic component having a structural role in chromosome behavior. That role, in meiosis, is associated with a distinctive pattern of semiconservative replication. The proportion of zygDNA sequences transcribed may be small. Our present estimates are that it is of the order of 1-2%. If the half-life of the transcripts is very short then that number may be an underestimate. Regardless of the precise value,

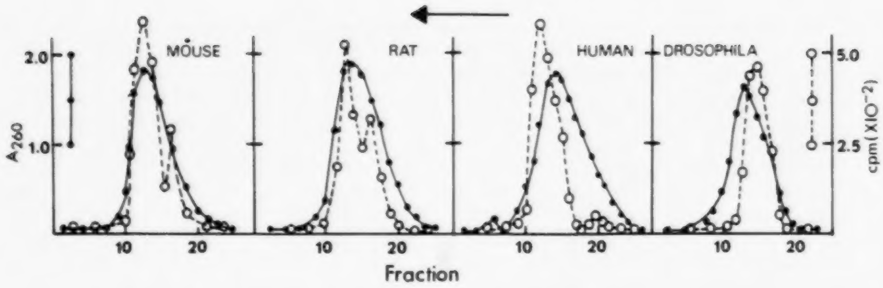

Fig. 4. Hybridization of lily zygRNA and DNA from several animal species. Each sample of DNA was centrifuged to equilibrium in CsCl gradient. Mouse and rat DNA samples were prepared from spermatocytes. Human DNA was prepared from placental tissue and Drosophila DNA was prepared from cultured embryonic cells. Each gradient fraction was tested for hybridization with 200 ng of lily zygRNA (1 x 10^7 cpm/μg) under moderately stringent conditions. The significance of the strongly asymmetric distributions of the hybridization profiles in humans and in Drosophila is unknown.

it has been established that all the zygRNA sequences identified are transcribed from DNA sequences that are replicated at zygotene as are all other zygDNA sequences. We do not know whether the sequences transcribed constitute a special subset of zygDNA segments that function solely in transcription and thus differently from the sequences presumed to be involved in alignment. Moreover, we have yet to establish whether the zygRNA transcripts are translated and, if so, how the translation products function.

The importance of a meiotic activity observed in lily is likely to be given greater significance if it can be shown to occur in more commonly studied organisms, particularly if they are phylogenetically distant from Lilium. This is indeed the case for zygRNA. When tested under conditions of moderate stringency, zygRNA hybridizes with DNA prepared from mammalian species on the one hand, and Drosophila on the other (Fig. 4). It also hybridizes with DNA prepared from Saccharomyces cerevisiae, Caenorhabditis elegans, and Sordaria.* It does not hybridize with prokaryotic DNA or DNA from mitochondria or chloroplasts. The results suggest that the sequences transcribed from zygDNA have a widespread phylogenetic distribution.

Much more fundamental evidence for the conservation of transcribed zygDNA sequences across the phylogenetic spectrum has been obtained from studies of mouse spermatocytes (5). Using lily zygDNA as a probe, zygRNA was found in poly(A)RNA extracts from prophase spermatocytes, but not in extracts prepared from either liver or bone marrow cells. Mouse zygRNA displayed a specificity for meiosis similar to that displayed by lily zygRNA. Using peripubertal and adult male mice as sources of spermatocytes it was further shown that the zygRNA was absent from the premeiotic spermatocytes as well as from the postmeiotic spermatids. As in lily, the major concentration of zygRNA was in meiotic prophase. The separation of meiotic stages that is achieved with lily is beyond experimental reach in the case of mouse spermatocytes so that no detailed profile of zygRNA concentrations during meiotic prophase is available.

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* We are grateful to Drs. Judith Kimble and Denise Zickler for the respective DNA samples.

It has been possible to extend the comparison between lily microsporocytes and mouse spermatocytes so as to demonstrate a zygDNA-like behavior for the mouse DNA that hybridizes with lily or mouse zygRNA. The key to this comparison is based on the evidence discussed much earlier that in *Lilium*, zygDNA can be excised at pachytene by treating isolated nuclei with S1 nuclease, the excisability being due to the incompleteness of zygDNA replication at zygotene (Fig. 2). If a similar situation exists in mouse spermatocytes it should be possible to excise selectively zygDNA at pachytene but not at the postmeiotic, round spermatid stage. Specificity of excision should be demonstrable by hybridization of the excised DNA with zygRNA. This is indeed the case. Nuclei isolated from prophase spermatocytes release a small amount of DNA following controlled incubation with S1 nuclease, the DNA hybridizing with zygRNA prepared either directly from lily or from prophase spermatocytes by passage through a lily zygDNA column. The bulk of spermatocyte DNA not so released showed negligible hybridization with zygRNA, as did DNA released by S1 nuclease from spermatid or from somatic nuclei. We consider these results as evidence for a widespread role of zygRNA in the meiotic process, and also for a widespread pattern of zygDNA replication during meiotic development. It thus appears likely that the role of zygDNA in synapsis is not a special feature of lily meiosis.

At present, we lack adequate information to integrate the two activities of zygDNA--delayed replication and transcription--into a single scheme that provides for a synaptic mechanism. As already emphasized, the evidence accumulated points strongly to a major role of zygDNA in meiosis. However, with respect to the particulars of synapsis, the only generalization that can be made is that at least several molecular events are required to effect homologous synapsis and that aneuploidy may be a product of a lesion in any one of these. It would be helpful to know whether chromosomes have a functional axis and whether this axis is defined by a specific class of DNA segments, such as zygDNA. If this were so, a more secure model might be advanced for chromosome pairing.

FUNCTIONS OF HOMOLOGOUS SYNAPSIS

Chromosome synapsis is commonly considered as an event directed at the juxtapositioning of chromosomes for effecting crossovers. The term has now been applied at the molecular level in referring to the sequence-specific positioning of DNA strands for subsequent recombinational activities. Quite apart from the fact that chromosome synapsis is not a magnified form of molecular synapsis, the accumulated evidence points to its having functions besides that of positioning homologs for crossing-over. In addition to the well-established evidence for the dependence of normal reductional disjunction on the maintenance of the paired association through metaphase, our observations point to a regulatory role of homologous synapsis on certain aspects of meiotic metabolism. The mechanism by which this occurs is unknown, but the consequence of its operation has major importance in the meiotic process.

Failure in homologous synapsis, whether a consequence of hybrid formation or a result of colchicine treatment, is accompanied by physiological changes in events that normally precede rather than follow crossing-over. It would appear that certain metabolic activities are regulated by synapsis; if the level of synapsis falls, so does the level of these activities (1). The behavior of R-protein is one example. Normally, it begins to rise in late leptotene-early zygotene, reaching a peak of activity during early pachytene. Its possible role in pairing has been considered earlier and its probable role in recombination has been pointed to elsewhere (11). This protein remains at a low level during the zygotene-pachytene stages in a diploid hybrid of lily that has a correspondingly low chiasma frequency. In amphidiploids, however, R-protein returns to normally high levels in parallel with the restoration of normal chiasma frequencies.

This particular example involves an elaborate regulatory mechanism affecting chiasma formation. A coordination of several events occurs during the zygotene-pachytene stages that leads to a programmed introduction of nicks in selected regions of DNA, such nicking presumably serving as initiating sites for crossovers (3). In the achiasmatic, diploid hybrid discussed above, the level of endogenous nicking also reflects the low level of homologous synapsis and chiasma frequency (1). The reduced nicking is not, however, a consequence of a depressed endonuclease activity. The latter rises and falls during meiotic prophase in the hybrid as it does in chiasmatic forms. Failure to nick is a consequence of a failure in the required alteration of chromatin structure that renders the selected DNA regions accessible to the endonuclease (3). Such accessibility is at least partly effected by the production of a group of small nuclear RNA molecules ("PsnRNA") and a particular protein S ("Psn-protein"). The RNA is homologous with the DNA at the sites affected, and the protein displaces the histones, thus rendering the sites accessible to the endonuclease. Both components are synthesized during zygotene and, in the case of reduced synapsis, both are formed at a correspondingly reduced level.

The mechanism responsible for maintaining the relationship between synapsis and the levels of certain metabolic components is unclear. It might be due to a very rapid destruction of components in the absence of synapsis. Alternatively, certain conditions required for initiating homologous synapsis may regulate the biosynthesis of these meiosis-specific components. In either case, synapsis demonstrably functions to regulate the expression of various metabolic events and not purely to juxtapose chromosomes for recombination. Some or all of these events may be directly involved in effecting recombination. ZygRNA which, as discussed earlier, is also present at a low level in absence of homologous pairing, may, if translated, have a direct bearing on some aspects of synaptic-dependent metabolism. As yet, we do not know whether the transcripts are translated.

The regulation and role of homologous synapsis in meiocytes extend from the premeiotic S-phase to postpachytene disjunction. The limited evidence available permits us to relate synaptic events to a particular set of DNA segments, zygDNA, to a protein with a specific binding affinity for a subset of zygDNA sequences, the L-protein, and to a set of metabolic events that are primarily expressed in late zygotene-early pachytene. It may turn out that in higher eukaryotes, at least, chromosome synapsis is not a process involving the alignment of homologous DNA sequences in general, but rather a process of aligning certain homologous sequences in particular. That process, let alone the accompanying one of stabilization, involves the coordination of several events beginning with the suppression of zygDNA synthesis by the L-protein followed by the activation of both zygDNA replication and transcription at the leptotene-zygotene transition. The incompleteness of zygDNA replication introduces yet another unanswered regulatory mechanism and an equally unanswered regulatory function. Even from a molecular standpoint, aneuploidy would appear to have several sources of origin.

ACKNOWLEDGEMENTS

The work discussed here was made possible by grants from the National Science Foundation, National Institute for Child Health and Human Development, and the American Cancer Society.

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DISCUSSION

MOENS: Yeast is unusual. Cells that have entered meiosis can still revert to mitosis. Those that reenter mitosis, in fact, already have synaptonemal complexes. Could you comment on the issue of commitment?

STERN: The character of meiotic commitment in higher eukaryotes is different. Upsets during meiotic prophase lead to aneuploidy.

RESNICK: Does the zygDNA inhibitory protein nick at a specific site on a specific strand?

STERN: We do not know. When the protein binds, only a small region is protected from DNase and only a single nick occurs on only one strand.

THE MEIOTIC PROCESS AND ANEUPLOIDY*

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INTRODUCTION

Meiosis occupies a central position in eukaryotic genetics because it provides the mechanism for precisely reducing the genetic material from the double amount present in the immature germ cell to the single amount present in the gamete. Deviations from the strict equality of distribution are almost always lethal or deleterious to the progeny, in humans as well as in Drosophila.

Many aspects of normal meiosis remain obscure or controversial. One fruitful approach to analyzing the process is to dissect it into its component parts, to analyze each part in as detailed a manner as possible, and then to attempt to reconstruct the whole. We recognize the principal meiotic events as (a) premeiotic DNA replication, (b) synapsis of homologs, (c) exchange of genetic material between synapsed homologs, and (d) segregation of homologs to opposite poles at the reduction division. While there is general agreement that replication and segregation mark the beginning and the end of the process, respectively, localization of the critical events of synapsis and recombination is still disputed.

The segregation mechanism came under scrutiny very early as the result of inconsistencies in each of the then current models. A basic Mendelian tenet holds that nonhomologs assort independently; yet through the introduction of rearrangements and aneuploidy it became possible to induce meiotic segregation between any two nonhomologs at will (15,30). Studies of this kind led to the Distributive Pairing Model of meiosis (16,23) which provides for the existence of a second pairing event that follows and is noncompetitive with genetic exchange. The second pairing, called distributive pairing, is limited to nonexchange chromosomes, is independent of homology but is size-dependent, and, of special interest, promotes regular segregation of nonexchange homologs. This model has resolved a number of apparent inconsistencies and has provided a fresh approach to the understanding of meiotic chromosome behavior. It is discussed in greater detail below.

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A second outcome of these studies was the disclosure of a strict concordance between recombination and disjunction. As originally proposed by Darlington (7), exchange between homologs produces a physical link which assures their regular segregation. Induction of distributive pairing between nonhomologs is always traceable to failure of exchange between the two members of a chromosome pair. To reach a better understanding of synapsis and recombination and their relation to other processes, a study of the very early oocyte was initiated.

THE OVARIAL SYSTEM

The reproductive system of the Drosophila female consists of 2 bilaterally placed ovaries, each made up of 15-20 ovarioles or egg strings. An ovariole possesses a very small anterior region, the germarium, where the oocytes are formed, and a large posterior region, the vitellarium, where the oocyte grows and matures. The oocyte originates as part of a 16-cell cluster containing 2 pro-oocytes* and 14 nurse cells. The 16-cell cyst moves posteriorly through the germarium to become a Stage 1 egg chamber with 1 oocyte and 15 nurse cells. It enters the vitellarium as a Stage 2 chamber, and at Stage 3 the oocyte chromosomes condense into a small body, the karyosome, where they remain through Stage 12. The oocyte becomes mature at Stage 14, having received the contents of the nurse cells and increased in volume ~100,000 times (31).

THE APPROACH

The traditional model of meiosis, postulated by Winiwarter (39), adopted by Darlington in the 1930s, and adhered to by most cytogeneticists today, places synapsis at the zygotene stage and recombination at the pachytene stage of meiotic prophase, well after completion of DNA replication and at a time when chromosomes are fairly well-condensed. A barrier to studying these stages in Drosophila has been the intractability of the oocyte chromosomes to light microscopy with the result that descriptions of prophase staging are equivocal and contradictory. For example, pachytene is reported to have been reached by the third or fourth germarial cyst (5) or at vitellarial Stage 3 (2); it is said to occupy a period between vitellarial Stages 3-8 (8) or a period between a midgermarial stage and vitellarial Stage 8 (9).

A more satisfactory approach, and one that we have adopted, is to use the S-phase in the oocyte as a reference time to mark the interphase stage. Synapsis and recombination can then be related temporally to this well-recognized stage. The traditional model would receive support if synapsis and recombination were found to follow premeiotic-S; it would be refuted if they were found to be coincident with premeiotic-S.

A TIMETABLE OF EARLY EVENTS

The Pupal System

Initial studies used adult females to monitor the times of exchange and replication. They were found to be coincident within the limits of resolution of the asynchronous adult system (26). The "pupal system" was developed to obtain better resolution (see Ref. 22 for details). Briefly, this method utilizes a population of synchronously developing females to study the first or vanguard oocyte found within each of their 30-40 ovarioles. The first oocyte is identifiable for cytological study because it occupies the most posterior 16-cell cyst in each ovariole; it is recoverable for genetic study because it is the first egg laid from each ovariole. The first synchronous batch of eggs is obtained by restricting oviposition to the first 10-15 eggs/female.

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* Henceforth the term oocyte will be used to designate a pro-oocyte that exists in the germarium between ~132 and ~168 hr.

Formation of the First Oocyte

In females developing at 25°C, the first 16-cell cyst containing the first oocyte is formed at 132 hr ± 2 hr postoviposition (p.o.) (4,29), close to the time of pupation at 132 hr. Stage 1 is reached ∼36 hr later at midpupation. The Stage 1 chamber leaves the germarium and enters the newly forming vitellarium as a Stage 2 at ∼168 hr p.o. Eclosion occurs ∼36 hr later (204 hr p.o.) when the first oocyte has reached about Stage 5. It becomes a mature Stage 14 egg ready for oviposition ∼36 hr later (240 hr p.o.) (Fig. 1).

Time of DNA Replication

Three procedures were used to delineate the S-phase in the first oocyte sample. They are as follows:

Autoradiograms of Feulgen-stained whole mounts. Ovaries were removed at 6-hr intervals between 132 and 168 hr p.o., and incubated in [^{3}H]-thymidine for 1 hr. Autoradiograms showing label in the posterior 16-cell cyst of the ovariole indicated DNA replication in the cyst at the time of incubation. On the basis of over 1,800 ovarioles, the S-phase was localized to a 24-hr period between 132 and 156 hr p.o. (22).

Autoradiography and transplantation. Ovaries taken at 6-hr intervals between 132 and 168 hr p.o. were incubated in [^{3}H]-thymidine for 1 hr and then transplanted into the abdomen of adults where attachment occurred and development frequently continued. If, upon removal, vitellarial stages were present in the transplant, autoradiograms were prepared. The presence of label in the karyosome signified DNA synthesis in this oocyte at the time of incubation. Results obtained in this way indicated that the S-phase occupied 30 hr (132-162 hr p.o.) with maximal activity between 138 and 156 hr p.o. (10).

Autoradiography and electron microscopy. Results identical to those in the preceding paragraph were obtained with this method and are described in the following section. Based upon the results obtained with the 3 independent approaches, the S-phase is localized between 132 and 162 hr p.o. (Fig. 2).

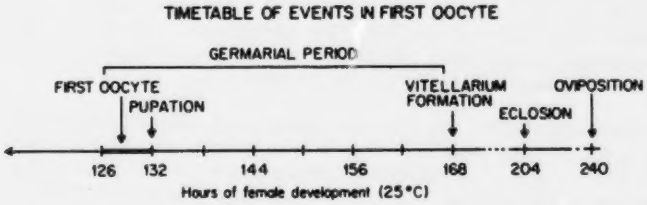


Fig. 1. Timetable of first oocyte development in females kept at 25°C.

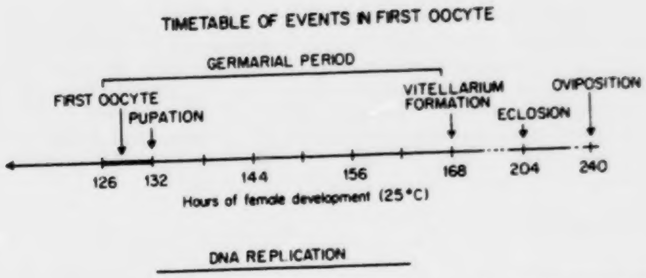


Fig. 2. Time of premeiotic DNA replication in the first oocyte.

Time of Synapsis

With the S-phase as a reference period, the initial appearance of a structure diagnostic of homologous synapsis called the synaptonemal complex (SC) was determined by electron microscopy. The results reveal a much earlier origin of the SC than had been previously assumed. At 132 hr p.o. the newly formed oocyte nucleus contains an extensive SC. Measurement of the SC in 9 oocyte nuclei, obtained from complete serial sections, shows it to average 50 μm at 132 hr p.o., or ~2/3 of its maximal length which is attained at 144 hr (29). At 132 hr, 55% of the first oocytes are found to contain SCs, whereas 2 hr later the frequency increases to 90% (29).

Figure 3 shows a longitudinal section of a very young germarium at 132 hr p.o. A single 16-cell cyst is present posteriorly, immediately behind the undifferentiated basal stalk, and a large stem cell is seen anteriorly. The oocyte nucleus (pon), indicated by an arrow, is shown below at higher magnification. A long SC lies between fairly condensed chromatin demonstrating that the SC is present at 132 hr p.o., about the time the oocyte enters premeiotic interphase.

By combining autoradiography and electron microscopy it was possible to follow germarial development and to demonstrate the simultaneous presence of SCs and label within the most posterior oocyte nucleus, beginning at 132 hr p.o., and at subsequent 6-hr intervals between 138 and 162 hr p.o. (29). Examples taken at 138 and 156 hr p.o. are shown in Fig. 4 and 5, and SCs between 132 and 168 hr are indicated in Fig. 6.

Time of Recombination

The time when recombination occurs in a eukaryote has long been a subject of inquiry and debate. The "pupal system" provides a well-synchronized oocyte sample that can be treated at specific, sequential periods to reveal the time of recombinational sensitivity. The agent chosen for treatment was heat which had been reported to increase recombination during a discrete period corresponding to the earliest oocyte (26,36). At the level employed, heat possesses the additional virtue of being a gentle, nonclastogenic agent that normally as an environmental variable helps to regulate the amount of exchange in Drosophila.

Treatments (35°C for 12 hr) localized the sensitive period for the total genome in the synchronized oocyte sample to 36 hr, beginning between 126 and 132 hr p.o., terminating between 162 and 168 hr p.o., and so being coextensive with S (Fig. 7). The coincidence was observed for >20 regions where significant increases were found. Regional asynchrony as to the time and degree of response was noted, but in no case was a response inducible after S. The peak response for the total genome occurs at 144 hr p.o.

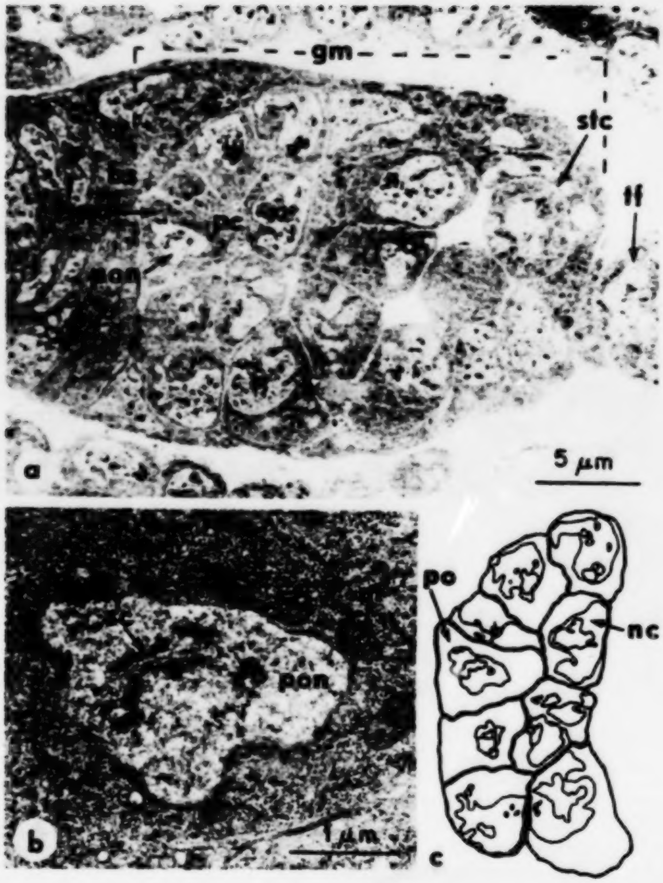


Fig. 3. Longitudinal section of one ovariole at 132 hr post-oviposition. (a) Very short germarium containing a single 16-cell cyst posteriorly and a large stem cell anteriorly. (b) High magnification of oocyte in 3(a) showing synaptonemal complex within oocyte nucleus. (c) Drawing of posterior 16-cell cyst in 3(a) with oocyte and 8 nurse cells. bs, basal stalk; gm, germarium; nc, nurse cell; pc, 16-cell cyst; po, oocyte; pon, oocyte nucleus; sc, synaptonemal complex; stc, stem cell; tc, terminal filament (from Ref. 29).

(27). As shown in Fig. 7 maximal SC length in the same oocyte sample is also attained at 144 hr, suggesting maximal exchange coincides with maximal chromosome extension. Further evidence for recombination during S comes from a study of the fourth chromosomes that normally do not recombine. Heat treatment induced low levels of recombination, but only if applied during S (19).

Recapitulation of Early Events

A timetable of the early events for the first oocyte sample is shown in Fig. 8. Oocyte formation at 132 hr ± 2 hr marks its entrance into pre-

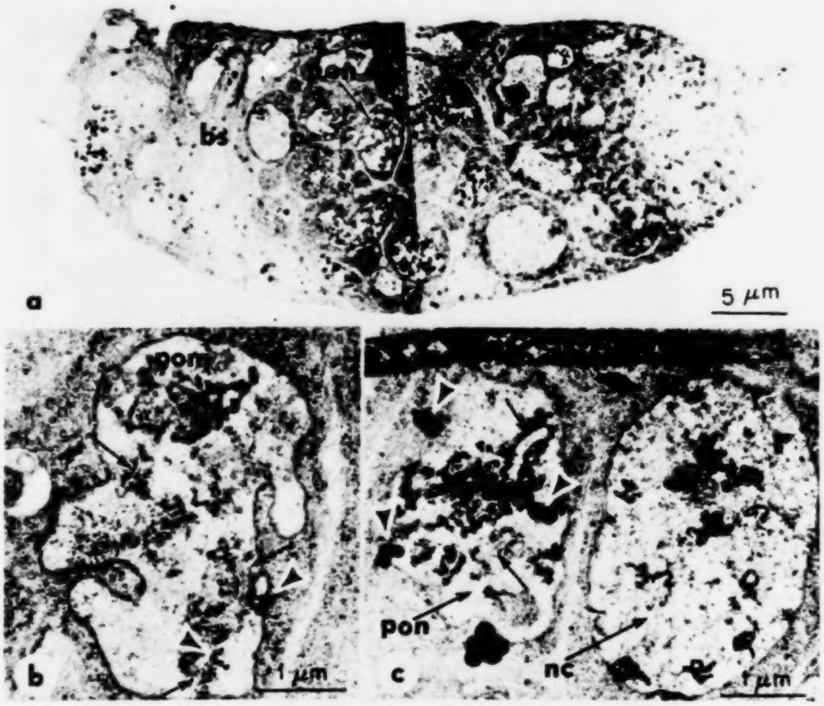


Fig. 4. Electron micrograph autoradiograph of longitudinal section of ovariole at 138 hr postoviposition. (a) Arrow indicates oocyte in posterior 16-cell cyst. (b) Higher magnification of oocyte in (a) showing label and SCs. (c) Oocyte showing label and SCs and labeled nurse cell from the posterior 16-cell cyst of another germarium at 138 hr. Arrows, SC; arrowheads, label; see Fig. 3 for abbreviations (from Ref. 29).

meiotic interphase. DNA replication begins at 132 hr and continues for 30 hr. Extensive SCs are present at 132 hr and continue into the early vitellarial stages. This finding permits the significant conclusion that homologs are synapsed and properly positioned for recombination during S. Finally, the period sensitive to enhancement and induction of recombination by heat begins close to 132 hr, terminates close to 162 hr, and hence is coextensive with S.

SEGREGATION

The Paradox

The relation of synapsis and recombination to segregation was vigorously pursued during the classical period of genetics. Chromosome pairing was generally conceded to be a concomitant feature of both exchange and segregation, yet no model emerged to causally relate the three events that was consistent with the genetic data.

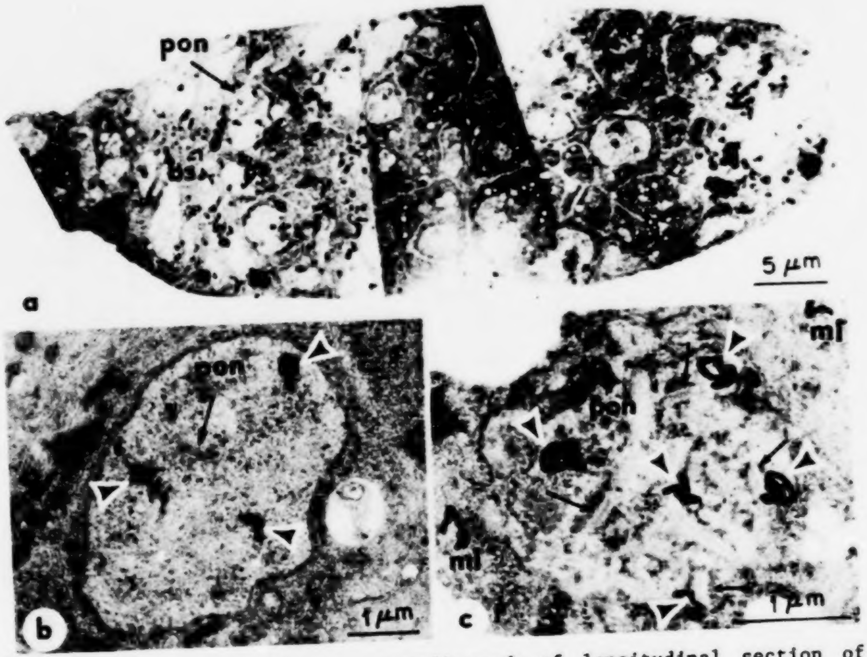

pon
5 µm
a
pon
b
1 µm
ml
pon
ml
c
1 µm

Fig. 5. Electron micrograph autoradiograph of longitudinal section of ovariole at 156 hr postoviposition. (a) Arrow indicates oocyte in posterior 16-cell cyst. (b) High magnification of oocyte nucleus in 5(a) showing label and SC. (c) Oocyte nucleus from posterior 16-cell cyst of another germarium at 156 hr p.o. showing abundant label and SCs. Arrows, SC; arrowheads, label; see Fig. 3 for abbreviations (from Ref. 29).

Darlington's hypothesis (7) proposed that regular disjunction of homologs is invariably dependent on chiasma formation and that every chiasma originates as a crossover. Yet with heterozygous X-chromosome inversions of Drosophila, where 80-99% of the Xs are estimated to be noncrossovers (6,12), or in the case of the small fourth chromosomes which are invariably noncrossovers, segregation remains extremely regular. Dobzhansky's (11)

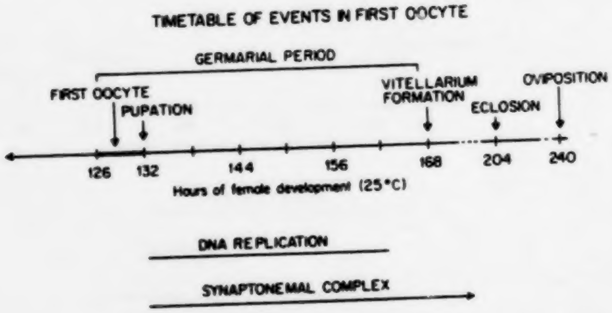

TIMETABLE OF EVENTS IN FIRST OOCYTE
GERMARIAL PERIOD
FIRST OOCYTE
PUPATION
VITELLARIUM FORMATION
ECLOSION
OVIPOSITION
126
132
144
156
168
204
240
Hours of female development (25°C)
DNA REPLICATION
SYNAPTONEMAL COMPLEX

Fig. 6. Time of synaptonemal complex formation in the first oocyte.

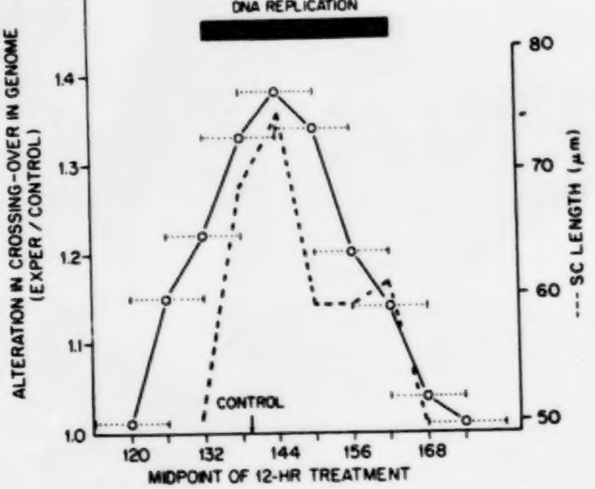
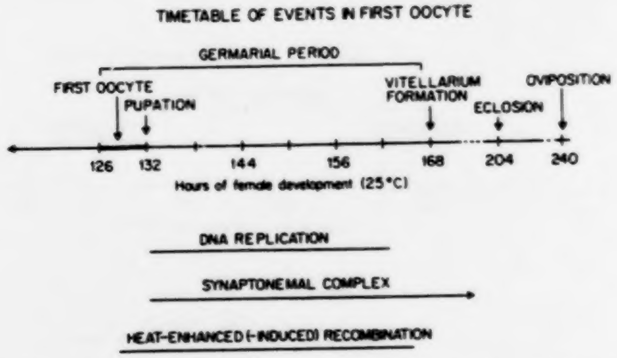


Fig. 7. Coincidence at 144 hr p.o. between maximal extension of the synaptonemal complex, maximal recombination response to heat, and mid-S phase in first oocytes from females kept at 25°C during development (from Ref. 29).

alternative hypothesis of "competitive pairing" assumed the critical feature to be intimacy of synapsis. Rearrangements that provoke a conflict between attraction forces in synapses were assumed to lead to decreased exchange and increased nondisjunction. But, as noted above with inversion heterozygotes, where synapsis and exchange are greatly disturbed, segregation remains highly regular.

Another puzzling aspect of segregation behavior became apparent with Bridges' model of secondary nondisjunction (3). Bridges noted that the addition of a Y chromosome to a female greatly increased X nondisjunction and that, as subsequently verified, nondisjoining Xs are drawn almost exclusively from the noncrossover X population (12,17,37). Bridges assumed that competitive X,X,Y pairing before exchange produced XY bivalents at the


TIMETABLE OF EVENTS IN FIRST OOCYTE
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144
156
168
204
240
Hours of female development (25°C)
DNA REPLICATION
SYNAPTONEMAL COMPLEX
HEAT-ENHANCED (-INDUCED) RECOMBINATION

Fig. 8. A timetable of the early events in the first oocyte.

expense of XX bivalents so as to create the noncrossover Xs that became secondary exceptions. A corollary of this model is an anticipated decrease in X-exchange proportional to the frequency of XY bivalents. Instead tests of this prediction revealed similar or slightly increased crossing-over with a Y (1). A second questionable aspect of the Bridges' model was the assumption that the Y, whose homology with the X was limited to a single known locus, could successfully compete with an X for synapsis.

Questions concerning the role of homology in synapsis came to a head with the discovery that, contrary to the law of independent assortment, nonhomologous chromosomes would segregate from one another with great regularity. This phenomenon was first demonstrated in females carrying a Y chromosome and a single free fourth chromosome (14,15) where it was found that in over 90% of the gametes, if the Y was present, the 4 was not; or, conversely, if the 4 was present, the Y was not; and that the two types of gametes occurred with equal frequency. Further, it was shown that under appropriate conditions, nonhomologous associations could involve any chromosomes or their parts and could reach frequencies as high as 99.9% (see Ref. 23 for review).

Initially the phenomenon of nonhomologous pairing added another ambiguity to the task of interpreting the relation of synapsis and exchange to segregation. The facts to be accounted for were the following: (a) Crossing-over is not a prerequisite for regular segregation, yet nondisjunctionals are noncrossovers. (b) A Y chromosome greatly increases X-nondisjunction, and produces no reduction in X-exchange, yet the additional nondisjunctionals are noncrossovers. (c) Pairing for exchange requires strict homology, yet, judging from segregation results, nonhomologous associations may occur with the greatest regularity.

Distributive Pairing

The traditional model of meiosis assumed that a single preexchange pairing suffices for both exchange and segregation. However, systems of late pairing between condensed chromosomes had been described by E.B. Wilson (38) and were recognized by H.J. Muller (34). Might the segregational pairing of nonhomologs in Drosophila fit this category?

A test of this possibility required an examination of the effect of nonhomologous pairing on exchange. Two dominantly marked nonhomologs, a noncrossover Y chromosome, and a multiply-inverted second chromosome, where crossing-over was limited by the inversion to the distal portion of the right arm (Fig. 9), were employed. The other second chromosome was in-

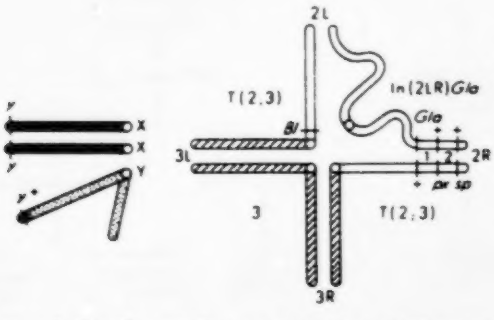

Fig. 9. Genotype of female used to test the single-pairing model (from Ref. 23).

volved in a reciprocal translocation with chromosome 3. Among the progeny of such females, 35% showed crossing-over in the noninverted portion of 2R. In these cases the Y assorted independently with the free 2. The remaining 65% possessed a noncrossover 2 and in these cases the Y segregated from the 2. This result demonstrated that nonhomologous association between Y and an autosome, like that between the Y and an X, is limited to noncrossover chromosomes. To answer the pivotal question, "are the noncrossover 2s that segregate from the Y produced by Y, 2 association?", crossing-over was compared in the Y-bearing females and in their otherwise genetically identical sibs which lacked a Y chromosome and hence lacked nonhomologous associations. Crossover values were found to be virtually identical (16). The test revealed that nonhomologous association does not produce noncrossovers. Rather, nonhomologous associations are established after exchange and exclusively between chromosomes that have failed to undergo exchange.

This information was incorporated into the Distributive Pairing Model of meiosis (16) (Fig. 10). Chromosomes that share complete or partial homology undergo exchange pairing. If exchange follows, homologs remain associated by a chiasma(ta) and segregate regularly at anaphase I. If they fail to exchange, or if the exchange occurs between the arms of a compound chromosome (isochromosome) (13), such chromosomes, as well as any that lack a homolog for exchange pairing, form the distributive pool of chromosomes which may then participate in a second pairing. Distributive pairing, unlike exchange pairing, can occur between nonhomologs as well as between homologs. Homologs that pair distributively segregate regularly; nonhomologs that pair distributively produce abnormal segregation patterns, namely, nondisjunction of homologs and regular segregation of nonhomologs.

The distributive model fits the seemingly irreconcilable data into a logically consistent sequence. For homologs that crossover, regular segregation is assured. In organisms where absence of exchange is frequent (due to inversion polymorphism, small chromosomes, or sexual dimorphism), nondisjunction need not be the consequence. The distributive system provides a second opportunity for nonexchange homologs (such as the X or 4th chromosomes) to segregate regularly. When distributive pairing occurs between nonhomologs, nondisjunction of homologs will follow and these will be the same noncrossovers that would have segregated regularly in the absence of

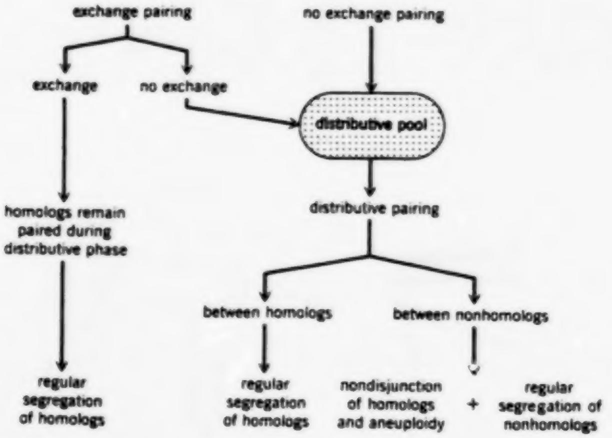
exchange pairing
no exchange pairing
exchange
no exchange
distributive pool
homologs remain paired during distributive phase
distributive pairing
between homologs
between nonhomologs
regular segregation of homologs
regular segregation of homologs
nondisjunction of homologs and aneuploidy
+
regular segregation of nonhomologs

Fig. 10. Distributive model for meiosis in Drosophila females (from Ref. 21).

the nonhomolog. Finally the requirement for strictly homologous preexchange pairing is maintained while the occurrence of a postexchange, distributive pairing with entirely different attributes accounts for nonhomologous segregations. There is reason to believe that a similar system may exist in man (20).

Attributes of Distributive Pairing

A clue to the specificity of distributive pairing came from a study of 2 chromosomes, a 4 and a T4 element (part of a 3:4 translocation carrying the centromere and heterochromatic base of 4 plus most of the right arm of 3). In the absence of a competitor, the quasi-homologs segregate 98% of the time; upon the introduction of a nonhomolog, the Y chromosome, the quasi-homologs assort independently while the T4 and Y segregate 98% of the time (30). Apparently homology of proximal heterochromatin plays no housekeeping role in segregational pairing. An obvious basis for preference might be size-similarity of the T4 and Y. To examine the role of size in distributive pairing, a series of X-duplications (deleted X chromosomes retaining only the tip and a variable amount of basal heterochromatin) that had been measured and assigned a size in relation to chromosome 4 (Cooper and Krivshenko, pers. comm.) was used. The duplications (Dps), whose sizes ranged from <0.3 to >4 times chromosome 4, were each introduced into genotypically identical females that carried a single free 4. In the noncompetitive situation, nondisjunction of the Dp and 4 is seen to be greatest with the largest and smallest Dps (Fig. 11, lower curve); to decrease as Dp size approaches 4 size (i.e., 1); and for Dps extremely close to the 4 in size (0.9 to 1.1), to average 0.1%, corresponding to a segregation frequency of 99.9%. In the competitive situation (Fig. 11, upper curve), the introduction of T4 (size ~5.5) as a competitor has no effect on Dp,4 nondisjunction when their size ratio is 2/3 or greater, since the two curves are virtually coincident for Dps of size 0.7 to 1.6. When their ratio falls below 2/3, there is a dramatic, symmetrical increase in Dp,4 nondisjunction imposed by this competitor. These results provide conclusive evidence that similarity of size regulates pairing at the distributive phase (18).

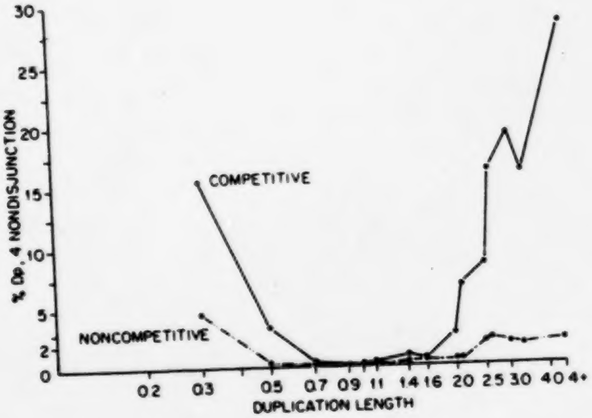

Fig. 11. Comparison of nondisjunction between X-duplications and chromosome 4 under competitive and noncompetitive conditions (from Ref. 18).

To determine whether arm length or total length defines size, Moore and Grell (33) substituted a compound-4 (two 4s attached to a single centromere to form a small metacentric) in place of a single 4. The predictions were for the compound-4 curve to coincide with that of the single 4 if arm length is the criterion; and to be displaced one unit to the right if total length is the criterion. As seen in Fig. 12, the compound-4 curve is displaced precisely one unit to the right in the noncompetitive and competitive situations demonstrating that total length determines size specificity. This evidence rules out a proposal (35) that a chromocentral formation, prior to synapsis and exchange, serves a segregational function by permitting chromosome tips to pair segregationally, corresponding to similarity of arm length as measured from a centromeric association.

CONTRIBUTIONS OF THE ts rec-1^{26} MUTANT

Recombination studies showed that heat response is coextensive with the S-phase during premeiotic interphase. If temperature acts directly, the sensitive period will delineate the time of recombination and place it at S. Temperature-sensitive mutants that permit normal expression at the permissive temperature and reduce or eliminate expression at the restrictive temperature are useful tools for localizing the time of action of a particular gene. When the restrictive temperature is applied at sequential intervals during development, the period during which wild-type activity is blocked identifies the active phase of the gene product. A temperature-sensitive recombination mutant could in this way mark the time of expression of a rec gene. A program to recover such a mutant was initiated and yielded the ts rec-1^{26} mutant, as well as 2 temperature-insensitive alleles, rec-1^{6} and rec-1^{16} (25). ts rec-1^{26} has provided additional information concerning the synaptic, recombination, and segregation processes.

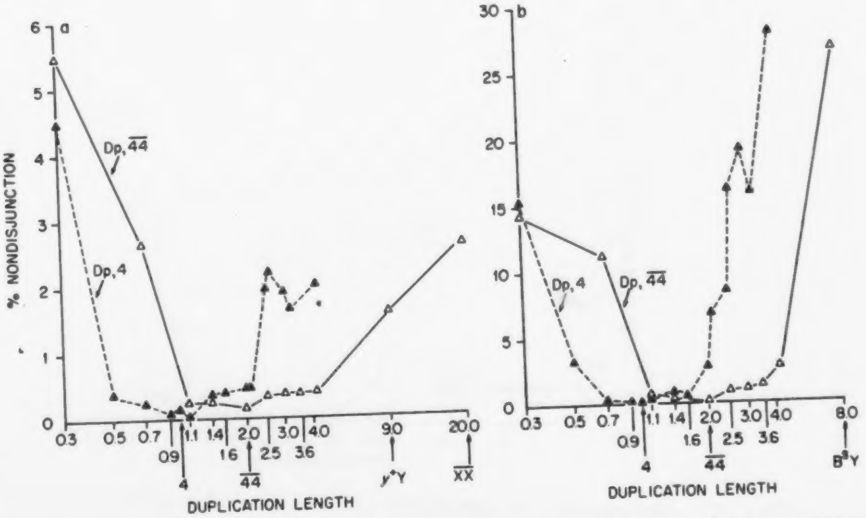

Fig. 12. Comparison of Dp,4 and Dp,44 nondisjunction under (a) competitive and (b) noncompetitive conditions (from Ref. 33).

Time of Action of *rec-1*^{26}

To determine when the restrictive temperature (31°C) affects recombination, synchronized females developing at 25°C were exposed to 31°C on successive days of development. Significant reductions occurred only on days 6 and 7 (120-168 hr p.o.) and were maximum when heat was applied on both days. By varying the treatment time during the 120 to 168-hr interval, sensitivity was resolved to a 36-hr period, beginning close to the time the oocyte enters premeiotic interphase and initiates DNA synthesis (126-132 hr p.o.), continuing for the duration of premeiotic-S, and terminating with completion of S at ~162 hr p.o. (24). Thus, acting in diametrically opposite ways, heat identifies the period of sensitivity for a reduction in exchange with the temperature-sensitive allele, and the period of sensitivity for the induction or enhancement of exchange in the normal genome, to be identical in time and both to be coincident with premeiotic-S (Fig. 13).

Synapsis and rec-1^{26}. The restrictive temperature could reduce recombination by affecting a *rec-1* product necessary for synapsis or a *rec-1* product involved directly in recombination. To determine if the target of the restrictive temperature is the SC, electron micrographs of serially sectioned Stage 1 oocytes, either heat-treated or untreated, were prepared. Stage 1 oocytes are formed very shortly after termination of the heat-sensitive period at ~168 hr p.o. The length of the SC measured 43 and 47 μm in the treated oocytes, and 43 and 45 μm in the untreated oocytes. No evidence of heat-induced changes in the fine structure or nuclear distribution of the SC was observed (28). Correlated genetic studies showed a reduction in recombination to ~10% of normal in the treated oocytes (28). This reduction in recombination is clearly not achieved through a loss or reduction of the SC, and is most simply interpreted to mean that the restrictive temperature acts directly on recombination. (The relation of synapsis to nondisjunction is discussed below.)

The Nature of the rec-1^{26} *Product.* Gene dosage studies show that *rec-1*^{26} is a leaky mutant with greater recombinational activity in 2 doses than in one (25). Activity of the mutant over a temperature range of 17°C to 31°C (Fig. 14) reveals a slightly negative slope between 17°C and 25°C, and a sharp decline between 28°C and 31°C (25). The sharp reduction within

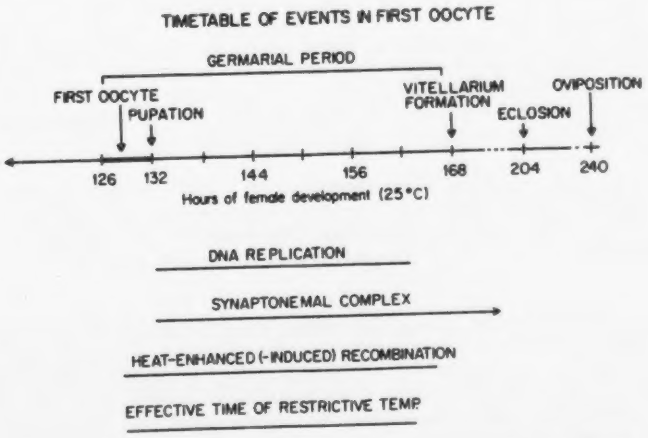
TIMETABLE OF EVENTS IN FIRST OOCYTE
GERMARIAL PERIOD
FIRST OOCYTE
PUPATION
VITELLARIUM FORMATION
ECLOSION
OVIPOSITION
126
132
144
156
168
204
240
Hours of female development (25°C)
DNA REPLICATION
SYNAPTONEMAL COMPLEX
HEAT-ENHANCED (-INDUCED) RECOMBINATION
EFFECTIVE TIME OF RESTRICTIVE TEMP.

Fig. 13. Effective time of restrictive temperature in first oocyte of *rec-1*^{26} females.

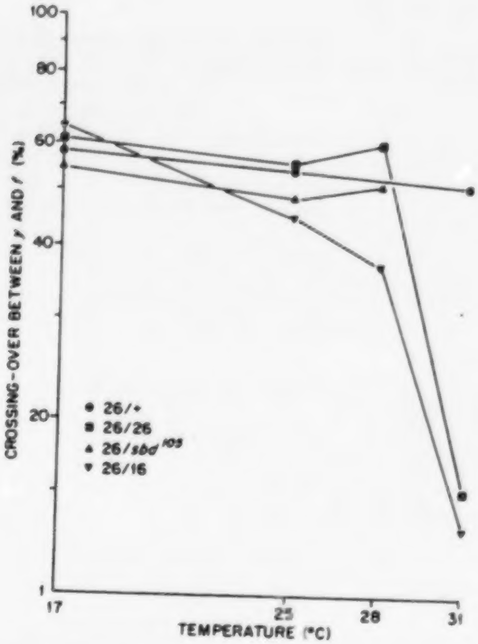

Fig. 14. Recombinational response of rec-1^{26} genotypes to increasing temperatures in the range between 17° and 31°C (from Ref. 25).

the narrow temperature range of 2-3°C is typical of a denaturation curve. It is reminiscent of the ts recA200 mutant of E. coli whose recombination activity is reduced more than one order of magnitude by a temperature increase of 2°C (32).

Apparently, then, the product of the rec-1 gene is a protein that is required for recombination and that is rapidly denatured by a 2° to 3°C increase in temperature above 28°C. Denaturation is expected to affect gene expression when the protein is participating in the recombination process and conceivably prior to the process, but should have no effect following recombination. Since the restrictive temperature has been shown to be ineffective after the S-phase, it follows that the involvement of the rec-1^{26} product with recombination is completed by the end of S.

Segregation and rec-1^{26}. Dobzhansky's view that synapsis bears the primary responsibility for ensuring regular segregation is still held by some geneticists. For example, Novitski (35) uses the following argument as the basis for his objection to the distributive pairing model:

> In the first place it seems inefficient for an organism, after having evolved an intricate mechanism which insures the synapsis of homologous chromosomes, to permit the synapsed chromosomes to fall apart freely (thus losing what must surely be one of the main benefits of synapsis, the apposition of homologues for metaphase orientation and normal disjunction), and would develop a second and different system (distributive pairing) to bring the chromosomes together once more, ...

Does synapsis in fact ensure regular segregation of homologs? An abundance of cytological evidence speaks to the contrary. A direct test at the ultramicroscopic level has been made with rec-1^{26}. The results show that under permissive and restrictive conditions, synapsis is indistinguishable; the SCs are identical in length, fine structure, and nuclear distribution (28). Does this mean that segregation under restrictive conditions is normal? On the contrary, there is an increase in X-nondisjunction from ~0.1% under permissive conditions to 20% under restrictive conditions. The 200-fold increase in nondisjunction rules out the notion that synapsis insures normal disjunction.

The rec-1^{26} mutant also provides information concerning the relation of recombination to regular segregation. Fig. 15 illustrates the effect of the restrictive temperature, applied on successive days of development, on X-recombination and X-nondisjunction (24). When heat is applied on days 2, 3, 4, 5, 8, and 9, recombination reaches control level and nondisjunction remains very low; heat applied on days 6 or 7 significantly reduces recombination and the reductions are accompanied by conspicuous increases in nondisjunction; heat applied on both days 6 and 7 produces the minimal recombination frequency and the maximal amount of nondisjunction. In short, a reciprocal relationship exists between the two processes.

Although recombination is measured in the X chromosome, there is good reason to suppose that the reduction extends throughout the genome. If the effect of the restrictive temperature were limited to the X chromosome, noncrossover X homologs would be expected to pair distributively, as they do in the case of heterozygously inverted Xs, and X-nondisjunction would remain very low The occurrence of high frequencies of X-nondisjunction implies the frequent presence of noncrossover autosomes in the distributive pool. Nonhomologous pairing between the X chromosome(s) and autosome(s) is responsible for both X-nondisjunction and the lethality arising from autosomal nondisjunction that is reflected in reduced fecundity (25). Studies

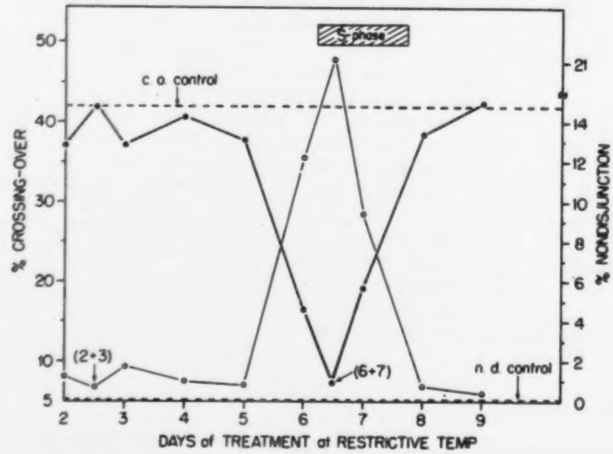

Fig. 15. Effect of restrictive temperature (31°C) on crossing-over and nondisjunction on different groups of developing rec-1^{26}/rec-1^{16} females heat-treated for 24 hr on sequential days of development from days 2 to 9, or heat-treated for 48 hr on days 2 and 3 or days 6 and 7. ●, crossing-over; ○, nondisjunction; ------, control; S-phase, premeiotic-S; c.o. control, crossing-over control; n.d., nondisjunction control (from Ref. 24).

have verified that conspicuous reductions in recombination occur in chromosome 2 with *rec-1* alleles (Grell, unpubl. results).

SUMMARY

Normal females developing at 25°C produce their first population of oocytes at 132 ± 2 hr post oviposition. Entrance of the oocytes into premeiotic interphase signals initiation of DNA replication which continues for 30 hr. Coincidentally, extensive SCs appear, averaging 50 µm (132 hr), peaking at 75 µm (144 hr), and continuing into early vitellarial stages. Recombinational response to heat, evidenced by enhancement or induction of exchange, is limited to the S-phase with a peak at 144 hr coinciding with maximal extension of the SC. Coincidence of synapsis and recombination response with S at premeiotic interphase is contrary to their conventional localization at meiotic prophase.

The interrelationship between exchange and nondisjunction has been clarified by the Distributive Pairing Model of meiosis. Originally revealed through high frequencies of nonrandom assortment of nonhomologous chromosomes, distributive pairing has been shown to follow and to be noncompetitive with exchange, to be based on size-recognition, not homology, and as a raison d'etre, to provide a segregational mechanism for noncrossover homologs. Rearrangements, recombination mutants, and aneuploids may contribute noncrossover chromosomes to the distributive pool and so promote the nonhomologous associations responsible for nondisjunction of homologs and regular segregation of nonhomologs.

Further information concerning early meiotic events and their relation to segregation has been revealed by studies of the *ts* *rec-1*^{26} mutant. Application of the restrictive temperature (31°C) at sequential times during development shows wild-type activity to be drastically reduced beginning at 126/132 hr, terminating at 162 hr, and so coinciding with S and the heat-sensitive period of the normal genome. EMs of serially sectioned oocyte nuclei maintained at the restrictive or control temperature reveal SCs to be indistinguishable, and implicate recombination rather than synapsis as the target of the mutant. Activity of *rec-1*^{26} in the range 17° to 31°C reveals a sharp decline between 28+° and 31°C, typical of a denaturation curve. If denaturation of the *rec* protein by the restrictive temperature marks its active phase, it follows that recombination terminates at the end of S when the restrictive temperature becomes ineffective. The notion that synapsis ensures regular segregation (35) is invalidated by *rec-1*^{26} whose normal synapsis at the restrictive temperature is followed by a 200-fold increase in nondisjunction at segregation, as compared to the control. The strictly reciprocal relationship between X-exchange and X-nondisjunction (Fig. 15) demonstrates that crossing-over is the controlling factor for regular segregation. Failure of exchange in the *rec-1*^{26} genome promotes distributive pairing between X and autosome univalents that is responsible for X-nondisjunction.

ACKNOWLEDGEMENTS

I wish to thank Drs. Ed Grell and Julian Preston for their critical review of the manuscript, and Nette Crowe for her invaluable help with the figures. Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-84OR21400 with the Martin Marietta Energy Systems, Inc.

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DISCUSSION

MCINTOSH: Can you exclude the possibility that there is a S-phase that precedes the labeling that you are seeing?

GRELL: Yes. We know the first pro-oocyte will appear shortly after the first synchronously dividing 8-cell cyst is observed at metaphase. Bucher (1957), on the basis of hourly inspections of ovaries developing at 25°C, found the first 8-cell cyst at 129 hr and our studies agree. The S-phase in embryonic and cultured somatic cells occupies 8-10 hr, and meiotic-S is much longer. So premeiotic-S could not have been completed and prophase attained at 132 hr when we found label and extensive SCs in the first pro-oocyte.

BERNSTEIN: A Robertsonian translocation between chromosomes 13 and 14 may increase trisomy 21 or X trisomy. In view of the size disparity between X and 21, how can the translocation induce both?

GRELL: Two acrocentric D chromosomes and the translocation will be present. The probability that neither will recombine with the metacentric is very small, but occasionally one D may be an univalent. If only two univalents are available, e.g., a D and a C and a X, they will pair and segregate with a high frequency. In fact, the first experiment demonstrating nonhomologous pairing in Drosophila utilized a large Y and a small 4 which segregated 92%, but a 4 and an X-duplication of the same size segregated 99.9%.

BERNSTEIN: What is your opinion of the chromosomal theory of Novitski which maintains that homologous and nonhomologous pairing occur at the same time?

GRELL: Novitski ignores the central evidence that the frequency of crossing-over remains unaltered by nonhomologous pairing. Any association of nonhomologs before exchange, chromosomal or otherwise, must reduce crossover frequency between homologs.

THE SYNAPTONEMAL COMPLEX IN MEIOSIS: SIGNIFICANCE OF INDUCED PERTURBATIONS

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INTRODUCTION

The synapsis of homologous chromosomes in meiosis is generally accepted to be a prerequisite for crossing-over. Moreover, there is evidence that both synapsis and crossing-over are determinants of the normal disjunction and segregation that lead to the reduction process by which haploid chromosome complements of germ cells are derived. It follows that perturbations in homologous synapsis are prime candidates for causing non- or abnormal disjunction and malsegregation of homologs, that could, among other consequences, lead to aneuploidy. But, visualizing the intimate morphological details of normal and abnormal synapsis, especially in mammalian gametocytes, is difficult at best with most commonly used cytological techniques.

Thus, available data about synaptic perturbations tell us little about the abnormalities themselves, but mainly concern the consequences seen in postsynaptic stages such as diplotene/diakinesis and first and second meiotic metaphases. Direct observation of synaptic morphology throughout meiotic prophase in normal and treated gametocytes could be the key to detecting alterations that precede such end effects as aneuploidy.

The synaptonemal complex (SC) is axial to the meiotic bivalent in spermatocytes and oocytes (11). It is a diagrammatically clear morphological correlate of synapsis. The dense, linear, proteinaceous axial elements of a pair of unsynapsed homologs come together during synapsis in meiotic prophase to form the lateral elements of the SC. They are joined together by central element material which includes fine, 2 nm transverse filaments. The fidelity with which the SC reflects the recognition of homology and the conjoining of parental chromosomes is the basis for the usefulness of SC analysis in investigating the details of the meiotic process, and in detecting perturbations in it. Moreover, it suggests that the SC provides conditions for and/or participates in the mechanisms that underlie synapsis and crossing-over themselves.

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These conclusions have been derived from studies using 2 types of techniques: (a) serial section reconstruction (20), which leaves the components of the nucleus fixed 3-dimensionally in situ, though an elegant technique, is tedious and not well suited to accumulating large amounts of data (cf., 20); and (b) the surface-spreading technique, which consists of underfixing hypotonically treated gametocytes and air drying them, while they are still soft, in the presence of a surface wetting agent (4), thus ameliorating artifacts of the drying process and producing essentially 2-dimensional structures from the flattened nuclei. Subsequent staining with silver permits observation by both light and electron microscopy. The results to be discussed below have been obtained with this technique.

FORMATION OF THE SYNAPTONEMAL COMPLEX AND STAGES OF MEIOTIC PROPHASE

The close parallels between synapsis and SC formation that make the 2 synonomous are evident from the normal behavior of the SC during meiotic prophase (Fig. 1). While neither the composition nor the details of assembly of the molecular components of the SC is known, the basic events of SC formation that delineate the stages of meiotic prophase are well documented (e.g., Ref. 9).

Leptotene

The filamentous axial elements assemble along the axes of individual homologs and apparently are formed at least partially under the direction of the chromosome. Assembly usually begins at the nuclear envelope.

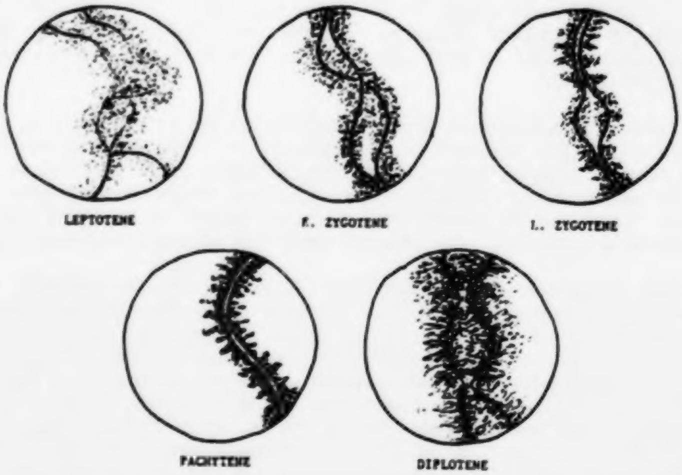

Fig. 1. Schematic diagram of autosomal synapsis and desynapsis in meiosis based on studies of spread preparations from 5 mammals. The essential structural components are shown: nuclear envelope, axial elements (chromosome axes), SC (with lateral and central elements), chromatin extending from the lateral (axial) elements, kinetochores on lateral (axial) elements, and terminal plaques by which axial and lateral elements are anchored to the inner face of the nuclear envelope. (From Moses [9] with permission of Academic Press).

Zygotene

Beginning of synapsis is marked by parallel alignment of homologous axial elements which, with central region material, form an SC, usually initiating at the nuclear envelope in mammalian cells, but not infrequently initiating interstitially. SC initiation is not synchronous among or along chromosomes, and axial assembly may occur in one region of a bivalent coincident with SC formation in another, more advanced region. While SC formation usually proceeds progressively from the initiation site, it does not necessarily do so by a continuous, zipper-like process; evidence suggests saltatory progression as though via a sequence of initiation sites.

Pachytene

This is the longest stage in meiotic prophase, lasting about 6 days in the mouse. Because synapsis (i.e., SC formation) is prerequisite for crossing-over, and pachytene is the stage at which synapsis is stabilized in the bivalent, it is often logically assumed to be the stage of crossing-over. But evidence showing that crossing-over can be inhibited or enhanced by treatments at other meiotic and premeiotic stages raises questions about when it occurs. One unifying concept is that crossing-over is consummated during pachytene. In this view, the other steps in the complex process of establishing and preparing a crossover site take place earlier, culminating during pachytene in the completion, or consummation, of a recombinational event that in turn leads to non-sister chromatid exchange and a chiasma figure at diplotene/diakinesis.

Diplotene

The end of the synapsed state in some species resembles a reversal of SC formation. In the mouse, the SC separates into single axial elements, held together here and there locally by residual short lengths of SC which are thought, from circumstantial evidence, to mark crossover sites. The axes then disassemble, attachment sites on the nuclear envelope being the last visible remains of the apparatus. In other forms (human, Chinese hamster), the lateral elements disassemble before they separate, while they are still part of formed SCs.

The foregoing description applies generally to autosome behavior, and to that of the 2 X chromosomes in the mammalian oocyte. The XY pair in the male is exceptional, synapsing partially, then desynapsing during pachytene. During that process, the axial elements often undergo elaborate morphological differentiations of unknown significance. The changes are so characteristic in a given species, moreover, that they can be used as markers of successive pachytene substages.

COMPOSITION OF THE SYNAPTONEMAL COMPLEX

The ideal biochemical approach to identifying the components of the SC would be to isolate the SCs, to extract the proteins, characterize and identify them, to raise monoclonal or affinity-purified antibodies against selected components to localize them cytologically, and ultimately to isolate the genes to produce quantities of the appropriate proteins and to reassemble them in vitro. However, there are presently technical obstacles to achieving these ends. Instead, in our laboratory we have chosen an immunocytological approach for our initial exploration, applying it to our whole mount spread preparations.

Preliminary observations using CREST (scleroderma) patients' sera containing antikinetochore antibody (Brinkley et al., this Volume) show that the differentiations along the SCs identified earlier on morphological grounds as kinetochores are bound by the antibody as expected (Fig. 11 in Ref. 14). These experiments also demonstrate that the immunocytological approach works on spread preparations.

An additional and more significant observation is that the sera of some patients also contain an antibody that binds to the SC. That this anti-SC antibody is distinct from the antikinetochore antibody is inferred from finding it in sera of some normal individuals, in the absence of antikinetochore activity. It was reasoned that counterparts might be found in strains of autoimmune mice from which monoclonal antibodies could be produced. This was indeed the case, and 2 related anti-SC monoclonal antibodies ("SC3" and "SC4") have been isolated. SC3, studied most extensively, binds selectively to formed SCs (Ref. 5; see Fig. 12 A, B in Ref. 14), but not to single axial elements, for example, at leptotene/zygotene or diplotene, or to unpaired axes of the XY pair. It also binds to the attachment plaques by which SCs and single axes are associated with the nuclear envelope, but not to kinetochores. As a rule other nuclear structures do not bind the antibody. Antibody labeling with colloidal gold for electron microscopy shows that this antibody binds to central region material, possibly the transverse filaments (5; see Fig. 12 C in Ref. 14). Although the protein itself has not yet been identified, it seems to be involved in formation of the SC, and hence is likely to be important in synapsis.

To learn whether the protein is specific to meiosis or is common as well to mitotic/somatic cells, the SC3 antibody was applied to cultured gerbil fibroma (CCL) cells. Here the antibody was found to bind to cytoplasmic filamentous material having morphological and behavioral characteristics of the cytoskeletal intermediate filaments formed from the protein, vimentin. Although antivimentin antibody did not bind to SCs, SC3 appeared to bind vimentin in Western blots. Continuation of these studies offers a promising lead to characterizing this and other SC-associated proteins, the first step in defining their role in synapsis, and by association, in disjunction.

SCHEDULE OF MEIOSIS

In order to use cytological observations on fixed tissues to discuss the progressive events of synapsis, it is first necessary to resolve the continuous process into a succession of easily recognized morphological stages. While the progression of morphological events in meiotic prophase can usually be inferred by arranging structural states in a reasonable sequence, there are often uncertainties to be resolved and duration of stages to be established.

In our mammalian spread preparations, we have taken the behavior and morphology of the XY pair (in the male), of the nucleoli, and to a lesser degree, of other more subtle, normal, progressive structural changes as morphological markers, defined with the electron microscope, for judging substages (11). By scoring frequencies of the different substages in large numbers of silver-stained nuclei with the light microscope, a time table of their relative durations from leptotene through diplotene has been derived, based on the assumption that frequency is proportional to duration (Fig. 2A). Then, from published estimates of the overall time span of meiotic prophase, the relative durations of the substages have been converted to approximations of absolute duration (Fig. 2A).

Because such approximations are subject to uncertainties in the underlying assumptions, they have been checked against ^{3}H-thymidine pulse-chase labeling experiments in which real-time data can be obtained.

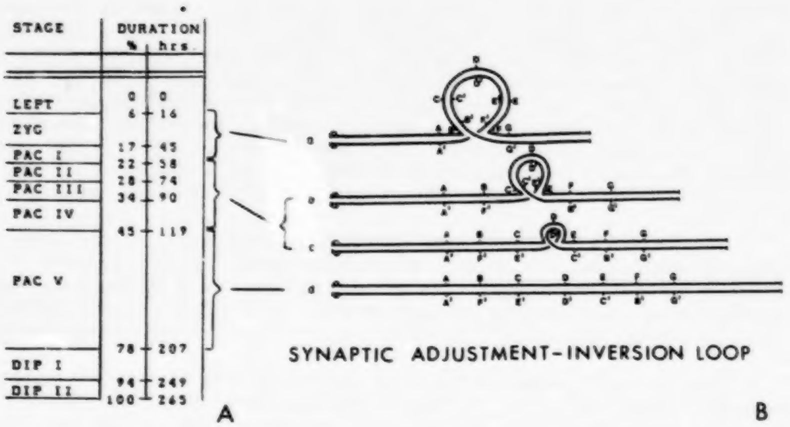

Fig. 2. A. Stages of meiotic prophase in mouse spermatocytes based on morphological criteria, and apportioned according to their cumulative frequency (%) in 1,000 cells scored. Insofar as frequency is proportional to duration, the data provide a relative time axis. Using the value of 265 hr from leptotene through diplotene (Oakberg [1957] Nature 180:1137-1139), an approximation of actual duration is derived (hr).

B. A schematic representation of synaptic adjustment in an inversion loop in the mouse is shown in relation to the stages and timing of meiotic prophase. A-G are arbitrary loci on the normal axis; A'-G' are comparable loci on the inversion-bearing axis. The inversion includes B'-F'. Homosynapsis is accommodated by the formation of the loop seen at early pachytene (a). Adjustment (loop reduction) occurs as homologous regions adjacent to the crux of the loop desynapse and immediately resynapse, but with a switch of pairing partners (b, c). Thus, in b, F and F', B and B' have desynapsed and with resynapsis, F becomes paired nonhomologously with B', and F' with B. As adjustment progresses, the loop is completely eliminated by late pachytene and replaced by "straight" SC (d), in which homosynapsis persists outside the inversion, while the region of the inversion itself is heterosynapsed. (From Moses et al. [15], with permission from Chromosoma, and Moses and Poorman [12] with permission of George Allen and Unwin.)

^{3}H-thymidine injected into animals is largely incorporated after 1 to 2 hr, and only germline cells in the S-period at that time will be conspicuously labeled. When the animals are sacrificed at sufficient successive intervals after injection, the meiotic stages that are labeled are determined autoradiographically and their progress through meiotic prophase followed with time.

Although premeiotic S-phase is generally thought to occur before zygotene, recent evidence has been taken to indicate that S-phase DNA replication in mouse spermatocytes occurs during the prophase stages characterized by SCs (6). To verify this point, a suspension of mouse testicular cells was incubated in ^{3}H-thymidine for 1 to 2 hr and fixed immediately thereafter; autoradiographs were then made of spread preparations. In such preparations, S-phase nuclei were easily visible by their heavy labeling; when examined by electron microscopy, none was observed to contain SCs. Furthermore, at the exposure times used, no nuclei containing SCs were appreciably labeled. However, occasional lightly labeled nuclei did contain the single axial elements of leptotene, or at most the beginning SCs of early zygotene, suggesting that premeiotic S-phase may terminate at, or overlap, the early stages. Our observations (13,14) thus do not support the contention that meiotic replication coincides with SC formation (synapsis). However, subsequent observations (reported below) using extended autoradiographic exposure times have in fact demonstrated a low level of DNA synthesis during meiotic prophase that is distinct from S-phase replication.

The in vivo incorporation experiments did confirm the morphological staging estimates. When the estimated timing of the labeled stages is plotted against the actual time after labeling, a straight line regression results (Fig. 3), indicating that within the limits of variability of the system, the morphological timetable is a reasonable approximation (14).

The in vivo experiments also confirmed the in vitro incorporation results. When the straight line is extrapolated to the abscissa, it intersects at approximately 24 hr prior to leptotene, indicating that incorporation, and hence S-phase DNA replication, occurs at that time. There was no evidence of any other substantial thymidine incorporation in the spermato-

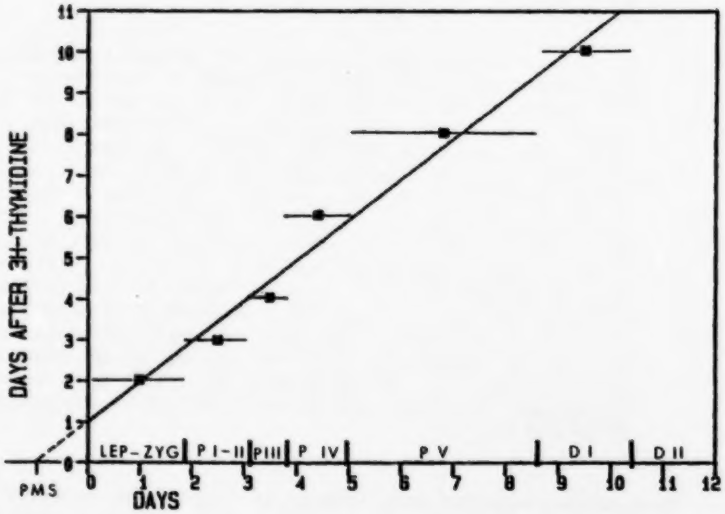

Fig. 3. The relationship between the time (in days; ordinate) after pulse labeling male mice in vivo with ^{3}H-thymidine and the staging of meiotic prophase based on duration of morphologically identifiable substages (in days, abscissa; calculated from frequencies; see Fig. 2A). Horizontal bars represent the duration (in days) of those substages most heavily labeled at 2, 3, 4, 6, 8, and 10 days after administering ^{3}H-thymidine. The solid squares are the midpoints of the substages. The curve of best fit to these points is a straight line, as shown, that intersects the ordinate at about 1 day and extrapolates to the abscissa at slightly more than -1 day, presumably the time of premeiotic S-period. These results validate the morphological time table and show that the S-period occurs prior to formation of the SC (zygotene).

cytes. The experiments were repeated with the Chinese hamster with comparable results. The inescapable conclusion is that there is a premeiotic S-phase that directly precedes synapsis and formation of the SC.

DNA SYNTHESIS ASSOCIATED WITH THE SYNAPTONEMAL COMPLEX

Biochemical evidence from the laboratories of Stern and Hotta, and of others, particularly from studies on lily (22) and mouse (7), has established that DNA synthesis of 2 kinds occurs during the synaptic period. Stern and Hotta (this Volume) have presented new information about the replication at zygotene of a small but distinct fraction of the genome that, together with an associated protein, is related to synapsis in lily. Other work, following lines laid down originally by Stern and Hotta (22), has shown synthesis during pachytene of a different small, distinct DNA fraction associated with a nicking/repair system that in turn is possibly related to homology recognition and crossing-over (23).

In the in vitro ^{3}H-thymidine incorporation experiments from our laboratory mentioned above, autoradiographs that had exposed in the dark for long periods of time (e.g., 26 wk) showed a small but significant amount of DNA synthesis in leptotene, zygotene, and pachytene nuclei of mouse and Chinese and Armenian hamsters (13,14). Moreover, at all stages, at least some of the label was associated with the SC, while at early-mid pachytene (e.g., substage P-III in the mouse; Fig. 2A), nearly all of the grains lay over SCs. It is important to reiterate that in no spermatocyte did the labeling ever approach the intensity associated with an S-phase nucleus.

From grain counts it became apparent that a stage-related, varying differential exists between synthesis of DNA associated with the SC and that in the chromatin at large (Fig. 4). Although there are marked dif-

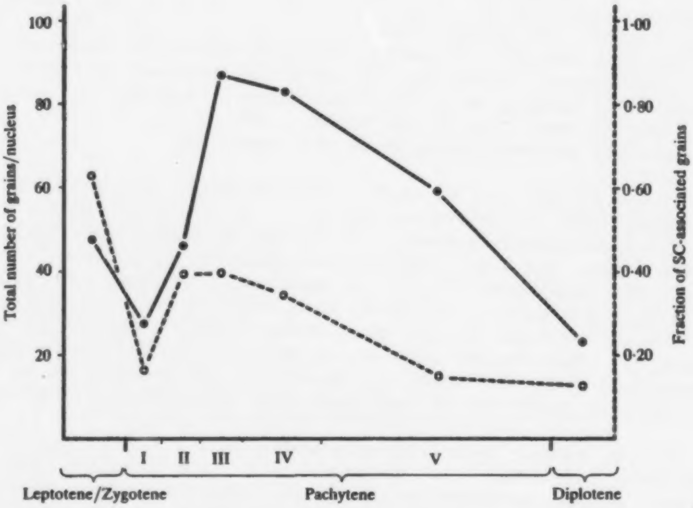

Fig. 4. Time course of ^{3}H-thymidine incorporation in mouse spermatocytes according to stage. Data are from differential grain counts of electron microscopic autoradiographs following in vitro incubation in ^{3}H-thymidine for 1 hr (15-20 week dark exposure). (From Ref. 14 with permission of The Company of Biologists, Ltd.)

ferences in the shapes of the time course curves of SC-associated DNA synthesis in the 3 species studied, in all 3 cases a distinct low occurs at the end of zygotene, followed by a high at early-mid pachytene, and then a slow drop into late pachytene and diplotene (Fig. 4).

DNA synthesis associated with the SC was also observed in preliminary experiments with mouse oocytes (12) in which hydroxyurea was found to block S-phase replication, but not pachytene incorporation, regardless of whether or not it was SC associated. This is the result that would be predicted if the pachytene incorporation represents the repair synthesis that has been demonstrated biochemically in mouse testis (7). It was not possible to determine whether the incorporation observed at zygotene included the DNA replication discussed by Stern and Hotta (this Volume). Nevertheless, our cytological results are entirely consistent with those of Stern and collaborators concerning zygotene and pachytene DNA synthesis, and relate them structurally to synapsis through the SC.

HOMOLOGOUS AND NONHOMOLOGOUS SYNAPSIS

The result of normal autosomal synapsis at zygotene is a bivalent comprising 2 homologous chromosomes closely paired, usually aligned homologously, chromomere by chromomere, and held in (possibly brought into) this configuration by a synaptonemal complex. The events at zygotene must involve: (a) some kind of localized recognition (which could have occurred earlier, possibly in the form of predetermination events); (b) initiation of synapsis/SC formation, presumably at recognition sites, coincident with or preceded by (c) drawing together of the homologs. As illustrated below, the progression of SC formation from an initiation site is probably more than simply a zippering process. Leptotene/zygotene in our preparations is seen to include mainly SC initiation and the preparatory events preceding it (i.e., axial element formation, accumulation of central region material, collocation of homologs). It also includes to a lesser degrees the events following initiation (i.e., continued SC formation). By contrast, zygotene includes mainly the latter. DNA synthesis drops from a high at leptotene/-zygotene to a low point at early pachytene, evidently decreasing as SC formation progresses. Thus, the DNA synthesis observed at leptotene/zygotene is related to homologous recognition, synaptic initiation, and/or SC formation, ceasing when the SC has formed. What then, if anything, does the pachytene DNA synthesis have to do with synapsis?

A clue is provided by studies of systematic perturbations of synapsis in rearrangement heterozygotes (11,14). In the first place, the qualitative and quantitative behavior of the SC in individuals heterozygous for translocations (14,16), duplications (11,14), deletions and insertions (8,14), and inversions (14,15) has so closely followed that predicted from cytological and genetic evidence, that these cases have provided the most convincing justification for considering synapsis and SC formation to be synonymous. The clarity and ease of detecting and characterizing such rearrangements form the basis for predicting SC analysis to be a practical and sensitive indicator of these and other chromosomal irregularities (10).

In the second place, these studies have provided new details about homologous initiation and synapsis, and have revealed that additional synaptic events, unsuspected in normal synapsis, occur during pachytene. It is to the latter that pachytene DNA synthesis may be related.

An inversion heterozygote in the mouse provides the best illustration of this point (15). At early pachytene, the inversion SC is seen invariably to have formed a perfect inversion loop. From the measured size and position of the loop, break points can be calculated, and these are found to agree with those determined from somatic banded kayotypes (15). Such observations underscore the regularity and precision of homosynapsis (and of the SC to reflect it), the loop being the topological means of establishing and maintaining it. To form such a loop, synaptic recognition/-initiation must take place at a minimum of 3 points: one on each side of the inverted region and outside of it, and one within it. SC configurations of early loop formation have been observed at late zygotene in both spermatocytes and oocytes that show this (11,12,15). The extent to which the SC conforms physically to accommodate homology is particularly evident at the initiation site within the inverted region where the 2 axial elements must reverse direction to maintain homologous pairing. It follows that recognition/initiation implies accommodation to polarity as well as site specificity.

The regular formation of inversion loops having the same size and with switchover of lateral element pairing partners at the expected inversion break points implies that homosynapsis from all 3 initiation points is complete. Occasionally, however, initiation at one end may fail, forming an incomplete figure with totally unpaired axial elements in one arm, and complete homosynapsis in the loop and in the opposite arm. In such cases synapsis does not appear to exceed the limits of homology, despite the opportunity to do so if a zippering process independent of homology were operative. Such findings support the general conclusions that at zygotene, synapsis is restricted to homologous regions, that heterosynapsis is prohibited at the stage where homosynapsis is operative, and that asynapsis is the only alternative permitted at this stage.

Standing in contrast to the above are the conclusions drawn from alterations of the inversion loop during later pachytene (12,15). While at early pachytene, loops are the rule, at late pachytene, all loops are replaced by "straight" SCs. Intermediate stages in the reduction, or "adjustment," of the loop at pachytene show that a switching of homologously paired SC lateral elements within the loop to nonhomologus partners occurs progressively at the switchover point at the base of the loop (see Fig. 2B), leaving heterosynapsed straight SCs in the inversion (Fig. 2B-d). While the reason for the inability of the bivalent to "tolerate" the homosynapsed loop is not clear, the fact of "synaptic adjustment" reveals a change in the control of synapsis from a restriction to homosynapsis at zygotene to an indifference to homology that permits heterosynapsis later on in pachytene. Further, it shows that synapsis can be undone and then redone independently of homology, but only after the restriction to homology has been lifted.

The phenomenon of synaptic adjustment, implying 2 phases of synapsis, has now been observed in a variety of rearrangements in the mouse (see references in Ref. 14), including pericentric and paracentric inversions, a tandem duplication, an insertion and deletion, and an indication that it occurs partially in reciprocal translocations. It also occurs with varying degrees of regularity in other organisms (lemur, rooster, sand rat, grasshopper, maize, etc.) and is probably a general synaptic phenomenon.

The behavior of trivalents in triploid Bombyx oocytes (19) fits the pattern of synaptic adjustment. At early pachytene, trivalents predominate, formed by the partial synapsis of 2 homologs with a third. Later, these are replaced by homosynapsed bivalents and univalents, presumably resulting from desynapsis of one homolog and the completion of synapsis of the other. Later still, bivalents predominate, some of them having been formed by the heterosynapsis of nonhomologous pairs of univalents. From these observations, Rasmussen (19) independently concluded that 2 phases of synapsis were operative, the later, heterosynaptic phase possibly providing a basis for distributive pairing (see Grell, this Volume).

When the process of synaptic adjustment, as for example of an inversion loop, is set against the time schedule of meiotic prophase stages (Fig. 2), correlations can be made with the DNA synthesis data which show that the peak period of SC-associated pachytene DNA synthesis coincides with, and thus may be related to, the period of adjustment. An interesting possibility in the light of Stern's observations (Stern and Hotta, this Volume) is that the drop in SC-associated DNA synthesis at zygotene is involved with the end of the homosynaptic phase, and that the increase in DNA synthesis in the first third of pachytene (repair?) is implicated in the shift to indifference to homology in synapsis.

One final point connected with synaptic adjustment concerns the timing of crossing-over. Rasmussen and Holm (20) believe that adjustment of the trivalents in Bombyx is a function of the absence of the crossing-over in the female, and that multivalents are not adjusted in the tetraploid male because of crossing-over. However this relationship does not hold in the mouse, as the presence of bridges at first anaphase indicates a substantial amount of crossing-over in the inverted region, yet virtually all inversion loops are adjusted. Furthermore, insofar as crossing-over depends on homologous synapsis, the essential event of crossing-over, or at least its consummation, must occur before adjustment is complete, i.e., before mid-pachytene.

While the observations outlined in the sections above provide no direct leads to the induction of aneuploidy in germ cells, abnormalities, induced or otherwise, in any or all of the systems discussed could conceivably influence disjunction, and hence figure in causing the defect. From a pragmatic point of view, however, the observations amply illustrate the accuracy and sensitivity with which SC analysis detects chromosomal perturbations that affect synapsis, and in particular the simplicity and ease with which it may be accomplished using the spreading method.

TESTING FOR THE EFFECTS OF MUTAGENS

The precise nature and extent of damage by mutagens to chromosomes in meiotic prophase, prior to diplotene, is not clear, the paucity of good documentation being due largely to the difficulties of analyzing conventional meiotic preparations. To test the effectiveness of SC analysis for assessing mutagen damage, 2 related agents were selected. Mitomycin C is a bifunctional alkylator, the primary action of which is known to be the cross-linking of DNA. Moreover, there is a large and varied literature on its effects on crossing-over, sister chromatid exchange, and other cytological and genetic indices of chromosome damage. Cyclophosphamide was tested because it is a monofunctional alkylating agent that requires systemic activation before it is effective, and because of reports that it induces aneuploidy (17,21). It is also an agent of interest because of its wide use in cancer chemotherapy.

From reports that mitomycin C produces rearrangements in spermatocytes (e.g., Ref. 1,3), we anticipated detecting them in our preparations. Instead, after intraperitoneal injections of either mitomycin C or cyclophosphamide, we discovered extensive SC damage in the form of breakage and fragment dislocation of unpaired axial elements, lateral elements, and whole SCs, and effects on pairing that included both asynapsis and heterosynapsis (18) (Fig. 5).

Of particular interest was the observation that with either agent, damage was not evident until several days after drug administration at the doses used (0.05-5.0 mg/kg for mitomycin C and 30-300 mg/kg for cyclophosphamide). Thus, severe damage was observed in zygotene/early pachytene

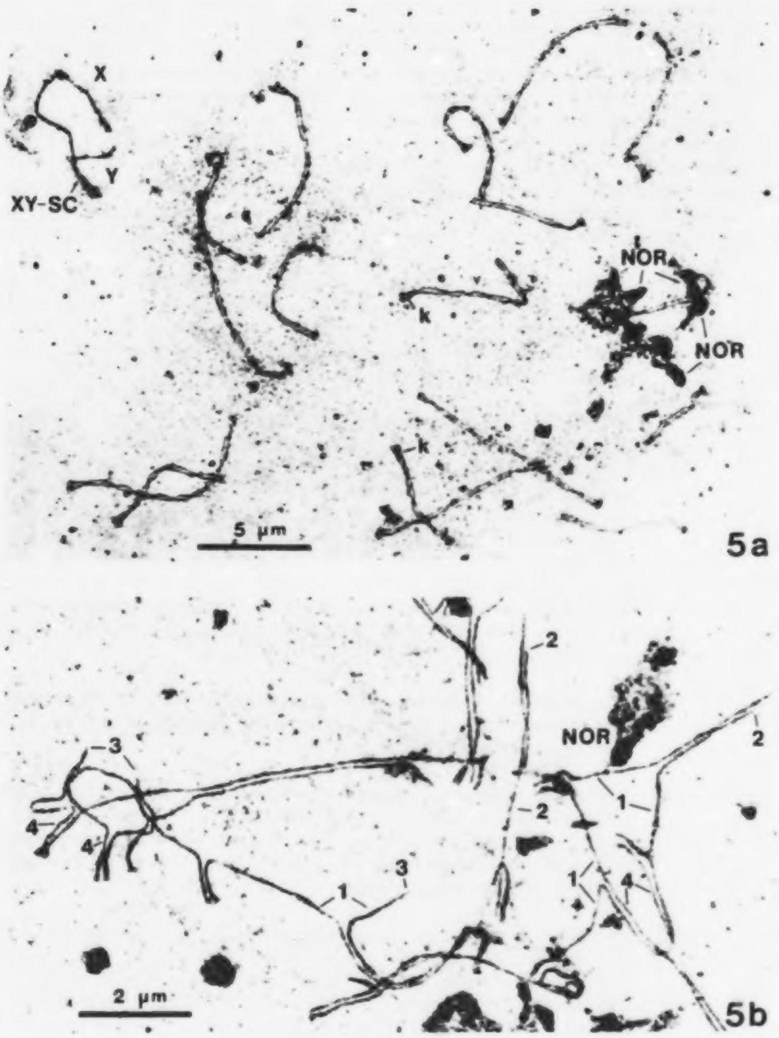

Fig. 5. Electron micrographs of portions of surface-spread, mouse spermatocyte nuclei at early pachytene, stained with silver to show synaptonemal complexes (SCs). a. Normal male mouse. The full SC complement consists of 19 complete autosomal SCs (only 17 are shown) and the XY pair. About half of the Y is paired with the X to form an SC (XY-SC). NOR = nucleolus organizer. k = examples of kinetochore regions. Bar = 5 µm. b. Mouse injected intraperitoneally 5 days earlier with 200 mg/kg cyclophosphamide. Severe damage to the SCs is evident as (1) asynapsis (single axial elements), (2) broken lateral elements with missing segments, (3) broken axial elements with missing segments, and (4) mispaired (heterosynapsed) lateral elements. Not shown are SC and lateral element gaps and displaced segments. NOR = nucleolus organizer. Bar = 2 µm.

nuclei after 5 days in the Chinese hamster, and after 3 days in the mouse. In both cases, the timing suggests that primary damage occurs during premeiotic S-phase. This inference was verified in the Chinese hamster, when ^{3}H-thymidine was given together with mitomycin C at time zero. At 6 days, the damaged cells were labeled while most undamaged cells were not. The most reasonable interpretation is that damage to the DNA by the agent occurs during the period of replication and produces a lesion that affects the integrity of the lateral elements and their ability to form homosynapsed SCs. This is the first firm evidence in support of the contention that SC formation is dependent on the integrity of the chromosome's DNA and raises the intriguing possibility that the sensitive DNA may also be that associated with the SC. Several other observations are significant:

(a) In the Chinese hamster, the SC-damaged cells can be tracked into pachytene but not through late pachytene; it is not clear whether the damage is repaired or the damaged cells are lost. Evidence from the mouse, where the cohort of damaged cells disappears at midpachytene, suggests the latter.

(b) Following the appearance of damaged cells at zygotene/early pachytene, a dropout of cells entering meiotic prophase indicates that an earlier cohort of cells was sufficiently damaged as to be unable to complete premeiotic S-phase. After a period of time, spermatocytes begin to reappear and re-establish a normal population that is apparently derived from surviving gonial stem cells. However, a low level of SC damage continues to be observed, even after 50 days in mouse as well as Chinese hamster, indicating that both drugs produce stem cell (gonial) damage which then persists in the germline and will probably continue to be expressed throughout the life of the individual.

(c) The degree of damage is dose dependent, in the range of doses known to produce other detectable damage, such as sister chromatid exchanges (e.g., Ref. 2).

(d) Higher doses of cyclophosphamide are required to produce results comparable to those with mitomycin C. These include SC disruption, failure of damage to persist through pachytene, drop out and then reappearance of subsequent cohorts of cells, and persistent gonial cell damage.

(e) Unlike mitomycin C, cyclophosphamide also induces a low level of SC damage at stages other than premeiotic S: particularly at leptotene/zygotene and early pachytene. The effect could conceivably be through interference with leptotene/zygotene or pachytene DNA synthesis, or by direct disruption of some intermediate process of SC formation.

Both mitomycin C and cyclophosphamide act at premeiotic S-phase to produce synaptic failure and SC (axis) fragmentation at zygotene/early pachytene. It follows that SC integrity and its attendant functions must in turn require normal DNA processing. By contrast, the primary site of action of colchicine is not DNA; yet when given at leptotene, it also interferes with synapsis/SC formation (J.B. Gibson; unpubl. results). Such perturbations of synapsis and SC organization by 2 aneuploidy-inducing agents, one (cyclophosphamide) a DNA cross-linker and the other (colchicine) interacting specifically with a protein (β-tubulin), suggests not only that the SC plays a key role in generating aneuploidy but that, as a target of potential agents that induce aneuploidy, its disruption could serve as a ready indicator for screening such agents.

The preliminary observations summarized in this section are obviously significant to an understanding of the SC, chromosome structure, synapsis, and crossing-over. But, of immediate and practical importance, they demonstrate a new, potential test system for screening chromosome damage in germline cells before they drop out of the system as a result of that damage. Furthermore, the test promises to be more sensitive than current cytogenetic assays (e.g., analyses at diplotene/diakinesis or at first and second metaphase) in revealing both transient damage (in the form of structural effects on the chromosomes through the SC, interference with synapsis, and cell death), and long-term damage (in the form of persisting effects in stem cells that lead to recurring SC and synaptic alterations). With respect to the latter, no currently utilized assay system, to our knowledge, has directly demonstrated cytologic damage to the germline after extended post-treatment intervals.

Studies are now in progress that are aimed at seeking correlations between the mutagen-induced SC damage discussed above and other end effects, in particular hypo- and hyperploidy at first and second meiotic metaphases, in sperm, and in early stages of zygote development. We also plan to extend the screening to other chemical agents.

ACKNOWLEDGEMENTS

The studies described were supported by research grants from the National Science Foundation (PCM-8303651) and National Institutes of Health (2P30CA14236), training grants from the NIH (MED and JBG; GM-7171 and GM-7754), and contracts from the Environmental Protection Agency.

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DISCUSSION

RESNICK: My question relates to the experiments in which you could detect repair synthesis. Perhaps these are the spots where recombination may be occurring. Do you have any evidence for or against whether that repair synthesis occurs in X and Y?

MOSES: There do appear to be patterns in the distribution of grains. Not only are there single grains along the SC, but also clusters which appear to be "hot spots." These might be the most interesting ones to map. Mike Dresser is interested in this question, but it requires developing a computerized system for recording and digesting grain distribution data.

EVANS: In many insects and plants there is good evidence for somatic pairing. Is there any evidence in these species?

MOSES: None that I know of. With respect to the somatic pairing and synapsis that occur in the early stages of salivary gland chromosome formation, for example, I know of no evidence for involvement of any special structural component. I would like to consider pairing to be something that somatic as well as meiotic chromosomes could do. The SC, as a chromosomal structure accessory to pairing, would extend it to the more precise and specialized process of meiotic synapsis, which includes crossing-over. In this view, formation of the salivary gland chromosome illustrates the precision and specificity of which the generalized chromosome pairing phenomenon alone is capable. That view may not take us very far, particularly with respect to how or why chromosomes pair, or how they recognize each other, but it may be worth considering.

HULTEN: I would like to make a comment leading up to a question about the detection of induced abnormalities using SC analysis. This could be readily studied in the human male if one could obtain testicular biopsies of men exposed to radiation where rearrangements would be predicted. I base this on our investigations of a number of Robertsonian and reciprocal translocations; they always form the expected multivalent in every cell that we have looked at. In one instance we were surprised at what appeared to be a reciprocal translocation in which we thought a hexavalent, rather than a quadrivalent, was formed. But we then found that we had made a mistake in mitotic analysis. From careful SC analysis, it was actually found to be a 3-break-point rearrangement. In another case we were surprised at the one inversion we have looked at: there was apparent nonhomologous pairing very early on. Rarely (only in 1 out of 159) was there an inversion loop. How quickly do you think an adjustment could happen? Could the fact that we are seeing hardly any inversion loops indicate that they are adjusting very rapidly, so that you get apparent nonhomologous pairing at the very first pachytene substage?

MOSES: All the mouse inversions we have looked at--singles or doubles--show either loops or asynapsis at early pachytene. On the other hand, a tandem duplication begins to adjust very early in some cells. I do not pretend to extrapolate from the mouse to all other organisms. The human is not a mouse. In my view, an animal model is not one that mimics the human, but rather is an experimental system from which one can extract principles and then look more economically to see if they apply in the human. The rule of thumb from the mouse is that if you look at enough inversion-carrying cells at late zygotene or early pachytene, you should find loops. If not, you must conclude that heterosynapsis in the human occurs early, and differs in detail from the mouse.

RISLEY: I would like to ask 2 questions about your antibody studies. It is known that there are structural domains shared between the intermediate filament proteins in somatic cells, and you have shown cross reactivity between your monoclonal antibody for the SC and vimentin filaments. But have you checked any other intermediate filament types?

MOSES: I will turn this question over to Mike Dresser who actually did the work.

DRESSER: We do not see labeling in frozen sections of muscle or neural cells, for example, indicating that the anti-SC antibodies do not cross react with desmin or neurofilament proteins, at least in these cells.

RISLEY: You have a monoclonal antibody that recognizes an epitope in SC proteins. It cross reacts with an epitope that is also in the intermediate filament proteins, but you do not see a reverse situation. If you were using a polyclonal antibody against the intermediate filament proteins, you might expect to see a cross reactivity. Was polyclonal antibody used?

DRESSER: No, but we have used a number of widely cross-reacting monoclonals. We are also aware of another point that is often brought up: many monoclonal antibodies cross react with intermediate filaments. Our observations are interesting mainly because there are also structural reasons for suggesting an association between the central region of the SC and the intermediate filaments. However, the connection cannot be made on the basis of these antibodies alone.

RISLEY: DiMartino has used immmunofluorescence to show the localization of myosin and actin in the SC. Have you tried to repeat that at the electron microscope level?

DRESSER: No, because we did enough experiments at the light microscope level to convince us that the myosin and actin results were probably a red herring, as indeed our own observations may well be. Others have also tried unsuccessfully to demonstrate actin and myosin in the SC. The problem is that in normal human sera, anti-SC antibodies are often present. Other workers say tubulin and MAPs are associated with the SC, based on the evidence that sera from rabbits immunized with those proteins also label the SC. But, what is binding the SC: the elicited antibody, or the resident anti-SC antibody? For example, we have an antitubulin serum from rabbit that also labels the SC. But then, when we do an affinity purification against tubulin for those antibodies, the affinity-purified antibodies do not bind to the SC, while the "left over" antibodies do. These are really tricky experiments and I do not believe there is at present a clear indication of any specific protein in the SC.

THE ROLE OF MOBILE DNA ELEMENTS IN UNEQUAL AND INTRACHROMOSOMAL CROSSING-OVER IN *DROSOPHILA MELANOGASTER*[*]

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INTRODUCTION

During the past decade the existence of mobile DNA elements, first deduced from genetic experiments in maize by McClintock, has been confirmed by their molecular cloning and characterization (3). In *Drosophila melanogaster* a parallel, albeit somewhat later, demonstration of the occurrence of mobile elements took place. The impetus of the genetic experiments stimulated a detailed molecular study of mobile DNA elements in *D. melanogaster*, the main points of which can be summarized as follows. More than 10% of the *D. melanogaster* genome consists of moderately repetitive DNA, the bulk of which is made up of mobile DNA elements. Several distinguishable classes of elements occur, the classification stemming from distinctive biochemical structures (for a detailed discussion, see Ref. 11). Considering the middle, repetitive DNA collectively and assuming mobile DNA elements average 5 kb in length and occur on the average in 25 copies per genome, the conclusion follows that approximately 50 discrete families of mobile DNA elements occur.

There is good evidence that mobile DNA elements are distributed more or less randomly over the *D. melanogaster* genome. A recent study (1), in which 12 discrete, cloned mobile elements were in situ hybridized to the polytene salivary gland chromosomes, revealed hybridization to ca 300 localized sites among a possible ca 2,000 sites. If this observation is representative of the general localization of mobile elements, and if the estimate of 50 discrete families of elements is correct, it follows that mobile elements are found in roughly half the possible chromosome sites. Irrespective of this estimate, it is fair to conclude that mobile elements are widespread in the *D. melanogaster* genome.

A substantial case can now be made for the causal association of mobile elements and mutation (11). As more and more genes are cloned, a consistent picture is uncovered: an inordinate number of spontaneous mutations (more than 50%) are causally associated with insertions of mobile elements. Thus the role of mobile elements in the spontaneous mutation process appears at this juncture to be established.

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[*] Research by author reported herein supported by National Institutes of Health grant GM22221.

But what of the role of mobile genetic elements in the generation of chromosome aberrations? Here the evidence is less extensive than the case for spontaneous mutation. Nonetheless, a case can be made and will be the subject of this chapter.

THE ROLE OF MOBILE ELEMENTS IN THE PRODUCTION OF UNEQUAL CROSSING-OVER

The phenomenon of unequal crossing-over in *D. melanogaster* is relatively "old hat," its occurrence having been deduced via classical genetic experiments by Sturtevant (12) with the X chromosome, dominant mutant Bar eye. He found that the "mutation" of Bar to wild-type or to the phenotypically more extreme Ultrabar was associated with marker gene exchange and crossing-over. This, he deduced, could only be explained by Bar associated with a tandem duplication. The "mutation" products were derived in females by unequal synapsis between the homologous duplications followed by a crossover within the synapsed region. Reversions to wild-type represented unduplicated chromosomes, ultrabar a tandem triplication. When polytene chromosome cytology became available, Sturtevant's deduction was confirmed.

Strictly speaking, however, the process by which Bar was originally produced is the unequal exchange event. Since the duplication is fairly long, involving the association in tandem of 6 polytene chromosome bands, it seems reasonable to suppose that nonhomologous pairing during meiosis accompanied by a crossover event could have generated the Bar duplication, and a reciprocal chromosome deletion equivalent in length to the region duplicated. Because the Bar "mutation" appears to have been an unique event, any speculation as to origin cannot be tested. Since its discovery in 1913 no subsequent occurrence has been found.

That unequal crossing-over as defined (production of a tandem duplication and a reciprocal deletion) can occur as a regular meiotic process stems from serendipitous findings during a study of genetic fine structure at the white-eye (w) locus on the X chromosome. When females of the genotype w^{a}/w^{a4} were progeny-tested, 2 unexpected classes of progeny were obtained (5,6). One class proved to carry a deletion of the two rightmost (centromere proximal) sites of the w locus. The second class proved to be the reciprocal direct tandem duplication, i.e., a duplication of the proximal sites. The deletion and duplication were recovered invariably with a concomitant, polarized gene marker exchange in a frequency of ca 1/5,000 progeny. The deletion, therefore, carried the right segment of the w^{a} chromosome and the left segment of the w^{a4} chromosome, the duplication carried the reciprocal segments, i.e., right segment of w^{a4} and left segment of the w^{a} chromosome. Three additional facts made this observation of unequal crossing-over puzzling. First was the finding that the w^{a} mutant itself was not essential for unequal crossing-over; what was essential was the genetic interval immediately centromere proximal to the w^{a} mutant. Thus the unequal crossovers were recovered in identical frequency from females w^{+}/w^{a4} where the w^{+} chromosome carried the region proximal to w^{a}. A second fact which confounded the situation was the observation that neither homozygous w^{a}/w^{a} nor w^{a4}/w^{a4} females generated unequal crossovers. A third confounding fact was the finding that among extensive heteroallelic combinations of w mutants studied by way of establishing their genetic mapping relationships, only w^{a}/w^{a4} combination generated unequal crossovers.

What seemed clear at the time was that production of unequal crossovers could be explained by the Bar model: asymmetrical pairing between the w^{a}- and w^{a4}-bearing chromosomes followed by a crossover to generate the deletion and tandem duplication. But how was the asymmetrical pairing brought about since the cytogenetics of the w locus uncovered no information suggesting gene duplication among any of the mutants studied?

This enigmatic situation remained unresolved for more than 25 years until it became clear that some mutants, in particular *w* mutants, were causally associated with the insertion of mobile elements (13). The following hypothesis was developed to account for the unequal crossing-over. Suppose a mobile element is integrated centromere proximal and immediately outside the *w* locus of the w^{a} X chromosome. Suppose, further, that a copy of the same mobile element is integrated in the *w* locus at the site of the w^{a4} mutation and therefore just distal to the two rightmost sites of the *w* locus. If pairing between the mobile elements occurs during meiosis and is accompanied by a single crossover within the paired elements, the crossover event generates a deletion of the rightmost sites and a reciprocal tandem duplication. It should be noted that this hypothesis fulfills specific requirements imposed by the empirical unequal crossing-over results. These are: (a) the crossover event is polarized so far as marker exchange is concerned, and (b) the w^{a} mutant per se has no part in the process.

The cloning of the white locus made possible the critical test of the aforementioned hypothesis. The molecular structure of the unequal crossover products established several crucial points (4). A mobile element, named Belshazar (BEL), ca 7 kb in length and *copia*-like in structure, can be localized in the predicted sites on the w^{a} and w^{a4} chromosomes. In the former BEL is localized just centromere proximal to the *w* locus; in the latter BEL maps to the w^{a4} mutational site. As predicted BEL is localized at the interface of the deletion generated by unequal crossing and, in addition, BEL can be localized within the reciprocal tandem duplication. Thus, all the requirements of unequal crossing-over being a function of pairing between copies of a mobile element are verified. The molecular distance separating BEL on the w^{a} and w^{a4} chromosomes approximates 60 kb. This means that the pairing affinity of familial mobile elements for one another suffices to overcome the strong forces which function to pair homologous chromosomes during meiosis. If pairing between mobile elements is the modus operandi, in general, for the production of tandem duplications, it follows from the genetic length of such duplications described thus far in *D. melanogaster* that pairing between familial mobile elements occurs over distances greater than the 60 kb found here.

It is fair to ask here whether the unequal crossing process described functioned in the production of other tandem duplications described in *D. melanogaster*. Unfortunately there is little data at hand, but there is at last one suggestion: the classical Bar duplication. Evidence is at hand which associates the Bar duplication with the mobile element, gypsy. The evidence stems from the finding that a specific suppressor mutation, *su(Hw)*, which suppresses a number of unrelated mutants in *D. melanogaster*, each characterized by the insertion of the gypsy element (9), also suppresses Bar. The inclusion of the gypsy element in Bar can be conveniently accommodated by involving the unequal crossing-over process described. Accordingly, the Bar duplication arose via asymmetrical synapsis between two gypsy elements juxtapositioned on homologous X chromosomes.

Finally, it should be noted that the unequal crossing-over process described here is not a phenomenon unique to *D. melanogaster*, but has been demonstrated to occur in all essential details in yeast via the Tyl mobile element (10).

MOBILE DNA ELEMENTS AND INTRACHROMOSOMAL CROSSING-OVER

Because of the intimate association between Mendelian gene segregation and meiotic chromosome disjunction, cytogenetic studies have centered in the main on the detailed analysis of the meiotic process. The method of genetics operates ipso facto by means of appropriate crosses in the recovery of the products of meiosis as gametes and ultimately as progeny. This method led to the discovery of crossing-over, gene conversion, nondisjunction, etc. in multicellular organisms. Because the meiotic genetic operation cannot be readily applied to mitosis, the genetic consequences of somatic cell division are not nearly as well understood as the genetic consequences of meiosis. Yet more than hints exist which demonstrate that mitosis is not a process which simply guarantees that the chromosome complement is transmitted qualitatively and quantitatively unchanged from one cell generation to the next in multicellular organisms. Where cytogenetic studies can be employed, it has been possible to demonstrate the occurrence of mitotic crossing-over and mitotic nondisjunction.

Cytogenetic evidence is on hand for the existence in *D. melanogaster* of a process of intrachromosomal crossing-over occurring during mitosis and mediated by mobile DNA elements. Part of the evidence for intrachromosomal crossing-over comes from the study of the fate of tandem duplications located on the X chromosomes of *D. melanogaster* males. Another part of the evidence comes from the fate of X chromosomes in *D. melanogaster* males whose genotype includes an autosomal *MR* element. These two parts will be discussed separately.

Tandem Duplications and Intrachromosomal Crossing-Over

The use of tandem duplications on the X chromosome of *D. melanogaster* males to assay for intrachromosomal crossing-over is premised on the following facts. *D. melanogaster* males have a single X chromosome and exhibit no meiotic crossing-over. Therefore, evidence for the occurrence of intrachromosomal, mitotic crossing-over can be inferred from the following genetic experiment. Males bearing an X chromosome tandem duplication are crossed so as to detect among their progeny individuals bearing a reversion of the duplication to the unduplicated or standard X chromosome sequence. The recovery of such individuals is presumed to take place by unequal crossing-over within one chromosome.

The actual experiment exploited the fact that the recessive X chromosome eye color mutant, zeste (*z*), is expressed in males only if males have two doses of the wild-type white (w^+) locus, e.g., a linked tandem duplication of w^+. The tandem duplication (Dp) selected was cytologically definable and sufficiently short so as not to impair male fertility (8). A breeding scheme was used in which *z Dp* males were crossed to double-X females. This breeding scheme directs the X chromosomes of males exclusively to their sons among whom a phenotypic change from zeste eye color to wild-type eye color can be objectively scored. Such a phenotypic change means that the two doses of w^+ in the paternal X are reduced to one.

The results of this experiment can be summarized as follows. Among the male progeny of the aforementioned cross, individuals with wild-type eye color occurred. The wild-type males occurred in clusters, i.e., from one male parent many wild-type sons are recovered. This means, in all likelihood, the change from two to one w^+ occurred premeiotically in a mitotically dividing spermatogonium, was increased in frequency by subsequent mitoses, and consequently appeared in more than one spermatozoan. The polytene chromosome cytology of individuals derived from several independent "reversions" demonstrated that in each case an exact change occurred from the tandem duplication to the unduplicated normal gene sequence. Thus 2 w^+ genes were reduced to one presumably by an intrachromosomal event.

What is the evidence for the involvement in the process of a mobile element? The evidence, for the moment, is entirely genetic. The presence of a mobile DNA element is inferred from the following genetic experiment.

When "reversion" males, wild-type in eye color, derived above, were crossed again to double-X females, there occurred among their progeny white-eyed sons in a frequency of ca 1/2,000 chromosomes scored. The occurrence of such white-eyed males was about 50 times the spontaneous mutation rate of w^+ to w. Subsequent objective genetic tests established that the white-eyed males were not the result of mutation, sensu strictu, but were the result of a small deletion involving the white locus. Extrapolating from the behavior of mobile elements in, e.g., *E. coli* and *D. melanogaster*, where such elements are associated with production of chromosome deletions, it seems reasonable to conclude that reversion chromosomes carry, juxtaposed to the w^+ locus, a mobile element which "excises" at a high rate and deletes a small chromosome segment including the w^+ locus. Since the duplication was generated originally in a male, it follows that the duplication carries 2 identical mobile elements, one adjacent to each w^+ locus.

The presence of 2 mobile elements, and the demonstrated potent meiotic synaptic affinity of familial mobile elements, suggest a model mechanism for the genesis of the reversions in males. Assuming that the mobile elements pair with each other in a loop-like configuration and a single-site, mitotic crossover occurs with the paired mobile elements. The intervening chromosome region is deleted resulting in the exact loss of half the tandem duplication and the precise restoration of the unduplicated, wild-type chromosome. This is diagrammed simplistically in Fig. 1. The idea of crossing-over between synapsed, linked mobile elements is not novel and has been observed to occur in the production of deletions in prokaryotes, yeast, and *D. melanogaster*, in association with a foldback DNA sequence associated with a mutationally unstable white mutant, w^c (2). What is novel is the distance over which pairing must occur to generate regular reversion of the tandem duplication. But since this event occurs in a mitotically dividing cell, restrictions in pairing between linked mobile elements are not influenced by the pairing forces which bring homologous chromosomes together in meiosis. Consequently, there is no a priori limit to the molecular distance over which synapsis between mobile elements can occur.

MR Elements and Intrachromosomal Crossing-over

Here it is relevant to consider quite another genetic system which illustrates that intrachromosomal crossing-over can occur in *D. melanogaster* males, such that nearly the entire X chromosome is deleted. The experimental set-up is as follows. In nature *D. melanogaster* flies carry in their genomes mappable, genetic elements originally described by Hiraizumi as *MR* elements (see Ref. 7 for a review). Such heritable, mappable exhibit elements have the following genetic properties. They increase the frequency of mitotic crossing-over in the gonial cells of *D. melanogaster* males and females. They also increase the mutation rate significantly either by directing the movement of P mobile elements to specific target loci or by generating deletions presumably by excision of P elements. They are themselves transposable. In many respects the *MR*-P interrelationship parallels the Ac-Ds interrelationship pioneered by McClintock for maize.

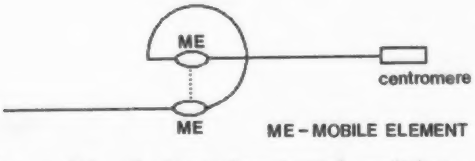

Fig. 1. A simple model of intrachromosomal pairing and crossing-over imposed by a mobile element pair. The point of crossing-over is indicated by the dotted line.

For quite an unrelated reason an experiment was undertaken in which double-X females homozygous for the recessive mutants yellow body color (*y*), white eye color (*w*), and forked bristles (*f*), were crossed to males bearing a wild-type X chromosome and an *MR* element in the chromosome 2, one of the large autosomes. It should be noted that the recessive mutants *y*, *w*, and *f* map genetically at 0.0, 1,5, and 52.5, respectively, on the X chromosome, the first near the telomere end and the last near the centromere end of the chromosome. It should be noted further that double-X females have two X chromosomes linked to one centromere which do not disjoin during cell division. And finally the double-X chromosome is deleted for the ribosomal region of the X chromosome and is lethal to embryos. Normally the Y chromosome ribosomal region which such females receive from their fathers rescues the double-X females.

In the aforementioned cross (double-X y w f/Y ♀♀ by +++/Y, MR + ♂♂), two unexpected classes of female progeny were recovered. One class included females of the phenotype *not yellow*, i.e., *y*$^+$, white-eyed, and forked bristles. A second class of females was phenotypically *not yellow* or *y*$^+$, red-eyed or *w*$^+$, and forked bristles. Each class of females occurred in a frequency of 1/3-4,000 female progeny and clustered. The *y*$^+$ *w*$^+$ *f* females exhibited the additional phenotypes associated with hyperploidy, were poorly fertile, and were not further studied. However the *y*$^+$ *w* *f* females were fertile and subjected to a detailed cytogenetic analysis. The results of this analysis involving 12 independently occuring *y*$^+$ *w* *f* females can be summarized as follows. All *y*$^+$ *w* *f* females carried a mini-X chromosome derived from the male parent, which included wild-type genes from both the tip of the X (and thus the *y*$^+$ phenotype) together with the base of the X chromosome plus the ribosomal region and the X centromere. Approximate genetic mapping of the mini-X chromosomes demonstrated that these mini-X chromosomes were generated by deleting most of the X chromosome. Based upon their genetic content, the mini-X chromosomes fall into a minimum of 5 different classes as illustrated by the mapping in Fig. 2.

Assuming that mini-X chromosomes are generated by an intrachromosomal crossover event as diagrammed in Fig. 1, and are mediated in part by synapsis between mobile elements located proximally and distally, 2 questions arise: Since a chromosome is made up of 2 chromatids, is the intrachromosomal crossover event an intra- or interchromatid exchange event? What mobile elements are involved in the intrachromosomal crossover process?

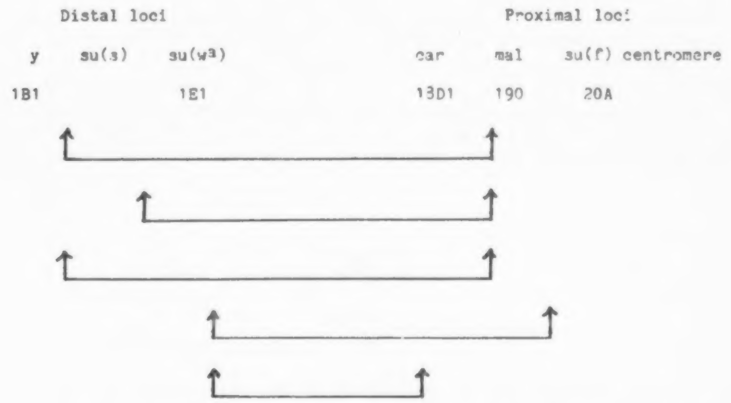

Fig. 2. Cytological limits of X chromosome deletions associated with the origin of mini-X. (Interval between arrows deleted.)

An answer to the first question can be deduced from experiments in which intrachromosomal crossing-over is sought in a ring-X chromosome. It has long been known that meiotic single crossovers are not recovered from females which are homozygous for a ring-X chromosome. Such a crossover leads to an interlocking ring which is not recovered in the progeny. If an intrachromosomal crossover is equivalent to a single crossover, then presumably an exchange between sister chromatids would generate an interlocking situation, while an intrachromatid exchange would lead to the production of a mini-X chromosome with a ring configuration.

An experiment was carried out to assess these possibilities: males whose ring X carried wild-type alleles at the proximal and distal loci, as well as an *MR* element on chromosome 2, were crossed to double-X females homozygous for the *y*, *w*, and *f* mutants. Among ca 30,000 female progeny scored, 2 y^+ *w* *f* and 8 y^+ w^+ *f* females were recovered. Of the 2 y^+ *w* *f* females found, one was fertile, one sterile. Subsequent breeding tests established that the fertile female included a mini-X chromosome carrying the distal y^+ and the proximal $su(f)^+$ genes. Mitotic chromosome cytology on the larval brain ganglia demonstrated the mini-X to be a mini-ring X. This finding is consistent with the idea that the intrachromosomal crossover event can occur as an intrachromatid exchange event.

Since *MR* elements are known to mobilize P mobile elements, it is inferred that these elements are associated with the genesis of mini-X chromosomes through intrachromosomal crossing. As many as 50 copies of P elements can be mobilized in the *D. melanogaster* genome. The genetic mapping of the mini-X chromosomes indicates that if the P elements are involved, they occupy at a minimum 3 different sites at the tip of the X chromosome: one site between the *y* and *su(s)* loci, one between *su(s)* and $su(w^a)$, and one to the right of $su(w^a)$. A comparable genetic situation occurs at the X base. When 4 independent mini-X chromosomes, each broken at the X chromosome base between the *mal* and *su(f)* loci, were genetically mapped, they were found to be broken at 3 different sites. Thus if the P sequences are involved in the intrachromosomal crossover event, and if the model proposed above is operative, it follows that the P mobile elements occupy a number of contiguous positions within one delimited chromosome region. This conclusion is supported by in situ hybridization localizations of P elements. When P elements are mapped on chromosomes isolated from flies collected in the wild, they can be localized, for example, to region 21 of chromosome 2, on some chromosomes to 21B, on others to 21D, and sometimes to sites simultaneously (Davis and MacIntyre, pers. comm.).

To sum up, a substantial case can be made for the origin of duplications and deletions by means of inter- and intrachromosomal crossing-over in which mobile DNA elements provide the sites of the crossover event.

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DISCUSSION

BOND: My question is with regard to zest-white. This is a particularly interesting locus because of the transvection effects. There are two white loci and they must be closely juxtaposed. Were there any events in which one of the white copies just moved?

GREEN: I have published a paper in *Molecular and General Genetics* this year in which I assumed that there must be two mobile elements in this tandem duplication and that, if there is one on each side of the white gene, then that makes it a transposition. Therefore, we should be able to make the white gene transpose, which it does, but with difficulty and less frequently.

BOND: You mentioned TYL elements in yeast and how they were analogous. They are like retroviruses in many respects. Do you think retroviruses are important in generating aneuploidy in man?

GREEN: This is a proposition to be taken seriously in the light of many of the rearrangements reported in cases of malignancy, particularly lymphomas and some of the cases found by Janet Rowley at the University of Chicago, and also the translocations studied by the Levan and Mittelman group in Sweden. There is a real possibility that they may be looking at juxtaposed oncogenes, which could be involved as foci. In the case of Burkitt lymphoma it is hard to understand how you get 8/14 translocations so frequently at the same place.

EVANS: This is not always true.

GREEN: But 8 is always involved, and there is more than one transposable element in the genome. So the possibility is not yet excluded.

EVANS: The relation of transposable sequences to cancer has been an exciting possibility for the last 3 years or so. The evidence, I am afraid, is against it, because the translocation that Dr. Green is referring to is the

8/14 in Burkitt lymphoma. The break points have been sequenced by a number of labs (Boston, Philadelphia, and Cambridge, England); they are not constant either in regard to the c-myc gene itself or to the immunoglobulin locus. In the sequences that are broken, nothing resembles a transposing piece of DNA. There is nothing that looks like a retrovirus; nothing that repeats at all in that sense. So unfortunately, there is so far no evidence in man for a transposable DNA sequence in association with these so-called specific translocations. Many years ago, Novitsky reported on a rather intriguing observation of a precise reinversion of an inversion. I guess that this would fall into Dr. Green's category of transposable element. Would you like to comment on this?

GREEN: I am not convinced; it takes more than one observation to make a generalization. I would like to see some other cases. About the reinversion situation, transposable elements may or may not be involved. But it is worth looking at. I can tell you about the Novitsky idea because I was in the lab when he did it. It involves a heterozygous inversion where the two chromosomes are synapsed into a big loop. Where the loops cross, there is a place, which if it is hit with X-rays, results in a complete reinversion. The observation has been repeated and it is correct.

HULTEN: Would you speculate on mobile elements in the human, for instance, with respect to the fragile sites on the distal part of the X chromosome that are commonly associated with mental retardation in the human population? There is some evidence that female carriers may have changed recombination.

GREEN: I would be reluctant indeed to ascribe all rearrangements to mobile elements. I cannot give you an answer for the fragile-X situation, or for the other fragile sites in the human genome. All I ask is that we keep open minds about the problem.

NONCOMPLEMENTATION PHENOMENA AND THEIR BEARING ON NONDISJUNCTIONAL EFFECTS

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ABSTRACT

In the mouse, unbalanced gametes with major gains and/or losses of chromosomal material seem just as capable of forming a zygote as normal, fully balanced gametes. This is shown by the results of intercrossing genetically marked translocation heterozygotes, in which complementary unbalanced gametes usually fuse to form fully viable zygotes. However, there are some notable exceptions to this. Studies on a number of reciprocal translocations have shown that gametes with maternal duplication of particular chromosome regions may fail to complement those with a corresponding paternal deficiency, but produce lethal zygotes instead, whereas the reciprocal combination of a paternal duplication with a maternal deficiency produces fully viable offspring. For a particular distal region on chromosome 7 the reverse situation holds. More recent studies on genetic methods of detecting nondisjunction with Robertsonian translocations have revealed the same phenomenon. Mouse chromosomes affected include numbers 2, 6, 7, and 8. There is also defective complementation on chromosome 11 and related phenomena on chromosome 17. These findings help to explain why diploid embryos with 2 male or 2 female pronuclei fail to come to term and may be connected with genetic imprinting of gametes. It seems probable that the same phenomenon occurs in homologous regions of human chromosomes and may mean that the severity of a trisomic effect will depend sometimes on the parental source of the extra chromosome. The phenomenon also affects the efficiency of certain genetic tests for nondisjunction which depend on full complementation.

INTRODUCTION

This paper is an attempt to show that when considering the harmful effects of aneuploidy it is necessary to look at the gamete as well as the zygote. Studies in the mouse have revealed that transmissible gametic aneuploidy can kill even when the resultant zygote is fully balanced. This finding raises the possibility that the phenotypic effects of particular aneuploidies may depend partly on the parental origin of the aneuploid gamete. It also throws light on the extent to which certain genetic tests for gains or losses of chromosomes can be used, and helps to explain why truly parthenogenetic embryos fail to survive.

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The fact that gametes with gross chromosomal imbalance can complement each other to give perfectly normal and viable zygotes has been one of the main pieces of evidence in support of the belief that the functional efficiency of eggs and sperm usually has very little to do with their genetic content. It has been nearly 40 years since Snell (39) first demonstrated this phenomenon with the T(5;8)a (now T(2;4)1Sn) translocation named after him, and further work on the subject has also concentrated on the behavior of translocations, both reciprocal and Robertsonian. Snell intercrossed heterozygotes for T1Sn, where one parent was homozygous for the recessive pallid (pa) on chromosome 2 and the other was homozygous for recessive brown (b) on chromosome 4, and obtained some brown pallid offspring when only wild-type would have been expected. The only logical explanation was that the translocation had generated viable gametes with a duplication of part of chromosome 2 and deficiency of part of chromosome 4 which had fused with complementary deficient, + duplicated gametes from the other parent to give normal offspring. Further work with other translocations (37,38) has confirmed this phenomenon, but has also shown that for certain chromosomal regions the expected complementation zygote does not appear, or dies prematurely. Mouse chromosomes implicated so far in this noncomplementation phenomenon are 2, 6, 7, and 8, with related phenomena on 11 and 17.

Before reviewing the evidence, however, it seems desirable to outline exactly what would be expected from reciprocal and Robertsonian translocation intercrosses, as the result of meiotic events.

CONSEQUENCES OF TRANSLOCATION INTERCROSSES

Reciprocal

Heterozygotes for reciprocal translocations produce about equal numbers of balanced and unbalanced gametes because of the reciprocal interchange of chromosome regions that has taken place (Fig. 1). Since the unbalanced gametes are usually associated with zygotic lethality in outcrosses, translocation heterozygotes are often described as "semisterile," though this is quite a different phenomenon from the true sterility found in male heterozygotes for certain translocations (35).

Let us assume (Fig. 1) a translocation occurs between 2 chromosomes, AB and CD, where A and C are the centromeric ends, so as to form the translocated chromosomes AD and CB. Then, by normal disjunction the resultant translocation heterozygote, AB.CD/AD.CB, will give equal numbers of the following gametes: AB.CD, AD.CB, AB.CB, and AD.CD. The latter two are unbalanced, with duplications of the distal region of one chromosome (B or D) and corresponding deficiencies of the other. If in an intercross of such translocation heterozygotes an AB.CB gamete fuses with an AD.CD gamete, then the zygote which results will be balanced, with the parental AB.CD/AD.CB arrangement. No less than 1/3 of balanced zygotes from normal meiotic disjunction in translocation intercrosses will be the result of fusion of complementary unbalanced gametes (38). If the distal arms are genetically marked with recessive-visible alleles, then the results of complementation can be detected easily if one of the parents is homozygous for the marker and the other is homozygous for its wild-type allele. Offspring that show the recessive phenotype rather than the wild-type will be the result of complementation. With normal complementation 1/6 of all viable intercross offspring would be expected to show the recessive phenotype if one of the two distal arms was marked. Thus, it is unnecessary to produce vast numbers of offspring in order to detect the presence or absence of complementation for genetically marked, distal chromosome arms.

Translocation heterozygotes also show low frequencies of adjacent-2

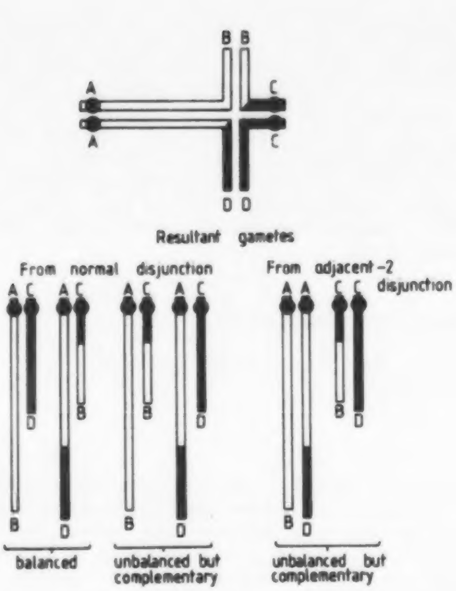

Fig. 1. Meiotic pairing and gametic products in a heterozygote for a reciprocal translocation, AB.CD/AD.CB, derived from 2 acrocentric chromosomes, AB and CD, by reciprocal exchange. For simplicity, the 2-strand stage with chiasmata is not shown, nor are all the products of adjacent-2 disjunction.

disjunction, i.e., nondisjunction of centromeric regions of the chromosomes, proximal to the translocation breakpoints. This leads to duplications and deficiencies of these proximal regions (Fig. 1), which can also lead to the fusion of complementary unbalanced gametes. The use of appropriate genetic markers allows the detection of this rarer phenomenon also, from which the frequency of adjacent-2 disjunction can be calculated and the location of centromeric regions of linkage groups can be determined (38). The frequency of such adjacent-2 complementation among viable progeny is unlikely to exceed 5% (12,38).

Robertsonian

Nondisjunction in heterozygotes for Robertsonian (whole-arm) translocations leads to gametes that are disomic or nullisomic for one or other of the chromosomes involved in the translocation. If these chromosomes are genetically marked, then the products of complementary nondisjunction can be detected in progeny of translocation intercrosses in the same way as with reciprocal translocations, and can be used to determine frequency of numerical nondisjunction in the heterozygotes concerned (22,25). Higher frequencies of nondisjunction are found in compounds of 2 Robertsonians with monobrachial homology (15) with respect to the common chromosome, so these are particularly useful for studies on nondisjunction of particular chromosomes (10,37). Unfortunately a number of such compounds are male-sterile (13), which prevents them being intercrossed to determine frequency of nondisjunction by genetical means. Where male-fertile, however, intercrosses can determine not only the presence or absence of successful complementation for the chromosome concerned, but can also allow comparison of the effects of different degrees of aneuploidy, e.g., trisomy vs tetrasomy. This is being done with respect to mouse chromosome 15 (5).

Although nondisjunction frequencies in Robertsonian compounds with monobrachial homology are high, as they are also in Robertsonians Rb1Bnr-Rb7Bnr derived from the tobacco mouse, the expected frequency of complementation is less than 1/5 of that with markers distal to breakpoints of reciprocal translocations. Moreover, many laboratory-derived Robertsonians have low frequencies of nondisjunction (14), so are unsuitable for complementation tests by intercrossing genetically marked heterozygotes. Thus, reciprocal translocations are best for locating regions of noncomplementation, while certain Robertsonians and their compounds are useful in determining whether particular whole chromosomes behave normally with respect to complementation.

EVIDENCE FOR ABNORMAL COMPLEMENTATION

So far, 6 chromosomes have been implicated and these will be dealt with in numerical order.

Chromosome 2

Four different reciprocal translocations involving chromosome 2 have been genetically marked, with heterozygotes intercrossed (36,39). Salient results (Tab. 1) show that (i) whenever the chromosome 2 distal marker was in the mother, marked offspring were not found or died around birth (*bp*); (ii) with one exception (T26H), whenever the chromosome 2 distal marker was in the father the marked offspring survived, usually at about the frequency expected; (iii) when the marker was on the other chromosome concerned (chromosome 4, 8, or 9) and carried by the father (so that it would appear in the offspring if there was complementation with a maternal gamete having a duplicate of the distal end of chromosome 2), then no complementation offspring were found; and (iv) the use of proximal markers led to low or zero frequencies of complementation-type offspring, as expected, because only adjacent-2 disjunction could give the necessary gametes with complementary proximal duplications and deficiencies. The translocation that proved an exception to these results was T(2;8)26H, since with both the chromosome 2 marker (*bp*) and the chromosome 8 marker (*e*), and with both maternal and paternal homozygosity, the expected complementation offspring died early or failed to appear. This aroused the suspicion, which proved well-founded, that the distal part of chromosome 8 was also involved in noncomplementation phenomena.

Chromosome 6

Our studies with this chromosome have been with the compound of 2 Robertsonians showing monobrachial homology for chromosome 6, namely Rb(6.15)1Ald and Rb(4.6)2Bnr, which gives over 30% nondisjunction (5). Some fertile males have been obtained (though many are infertile) and crossed to similar Rb1Ald/Rb2Bnr females homozygous for the chromosome 6 marker white, Mi^{wh}, which is a "mock-albino." This gene is particularly useful because the heterozygote (light with some white-spotting) can be distinguished from both homozygotes. These crosses have given 114 Mi^{wh}/+ progeny and 8 wild-type, but no Mi^{wh}/Mi^{wh} in 122 mice (5). This suggests that maternal disomy for chromosome 6 does not complement paternal nullisomy to give viable offspring, in contrast to paternal disomy combined with maternal nullisomy. This agrees with Cattanach's results (22) with intercrosses of Rb2Bnr heterozygotes on their own, in which the chromosome-6 marker was *wa-1*. When the father was *wa-1*/*wa-1* and the mother +/+, he obtained 10 waved-1 progeny in 1,113 mice, but in the opposite cross he obtained none in 948 offspring. Thus, there is good evidence that some locus or region on chromosome 6 leads to developmental trouble when 2 doses come in from the mother and none from the father.

Tab. 1. Results of intercrossing genetically marked translocation heterozygotes which involve chromosome 2. Marked chromosome shown in parentheses. See Ref. 36, 38, and 39.

Translocation	Marker	Sex of carrier (M/F)	# progeny classified	% marked in progeny	
				Observed	Expected
T(2;4)1Sn	*pa* (2)	F	32	0.0	$<5^b$
	pa (2)	M	292	0.7	
	b (4)	F	292	0.7	
	b (4)	M	32	0.0	
T(2;4)13H	*m* (4)	M	25	0.0	16.7
T(2;8)26H	*bp* (2)	F	61	3.3^a	16.7
	bp (2)	M	327	12.5^a	16.7
	pa (2)	F	24	0.0	$<5^b$
	pa (2)	M	226	2.7	$<5^b$
	e (8)	F	32	0.0	16.7
	e (8)	M	69	0.0	16.7
T(2;9)11H	*bp* (2)	F	107	12.1^a	16.7
	bp (2)	M	154	18.2	16.7
	a,a^t (2)	F	72	0.0	16.7
	a,a^t (2)	M	53	11.3	16.7
	fi (2)	M	104	1.0	$<5^b$
	cv (9)	M	47	0.0	$<5^b$
	se (9)	F	72	8.3	16.7
	se (9)	M	53	0.0	16.7

[a] Perinatal death instead of normal viability.

[b] Complementation visible only after adjacent-2 disjunction, since marker is proximal to breakpoint.

Chromosome 7

The first suspicion that normal complementation of unbalanced gametes was impaired in certain regions of chromosome 7 arose from the work of Snell (39) on a translocation he named *T(1;?)c*, which is now extinct, but can be called T(7;?)2Sn on present nomenclature rules. He intercrossed heterozygotes for this translocation, in which one parent was homozygous for the chromosome-7 marker, *sh-1*, and the other was wild-type, but found no shaker-1 offspring (Tab. 2). We now know that the *sh-1* locus was distal to the translocation breakpoint, which showed no recombination with the albino (*c*) locus (39) on chromosome 7, so 1/6 of surviving offspring should show the marker's phenotype. A second clue was provided by the apparent absence of any chinchilla (c^{ch}) pink-eyed (*p*) offspring from mutation experiments with the original 7 specific loci (31), which include c^{ch} and *p* on chromosome 7, although a number of dilute (*d*), short-ear (*se*) mice (*d* and *se* being included also and closely linked on chromosome 9) had been found which seemed to be the result of simultaneous nondisjunction rather than of a deficiency which covered both loci (11,32,34). One possible instance of double nondisjunction for chromosome 7 has been reported (33) at the *Hbb* locus on this chromosome, but there are other explanations. For

Tab. 2. Results of intercrossing genetically marked translocations which involve chromosome 7. Chromosomes marked are given in parentheses.

Translocation	Marker	Sex of carrier (M/F)	Total progeny classified	Freq. marker in progeny (%)		Reference number
				Observed	Expected	
T(7;?)2Sn	*sh-1* (7)	F	31	0.0	16.7	39
T(7;?)2Sn	*sh-1* (7)	M	80	0.0	16.7	39
T(7;?)2Sn	*p* (7)	F	80	0.0	<5[a]	39
T(7;?)2Sn	*p* (7)	M	31	0.0	<5[a]	39
T(7;15)9H	+,Ca^{d} (15)	F	89	0.0 +	16.7	4 and unpubl.
	+,Ca^{d} (15)	M	76	0.0 +	16.7	4
	uw (15)	F	35	5.7	<5[a]	4
	uw (15)	M	31	0.0	<5[a]	unpubl.
T(7;18)50H	*p* (7)	F	182	0.0	<5[a]	2
	p (7)	M	71	4.2	<5[a]	36

[a] Complementation visible only after adjacent-2 disjunction, since marker is proximal to breakpoint.

example, somatic recombination in an early cleavage stage heterozygote could lead to incorporation of only a homozygous clone in the embryo proper.

Results with both T(7;15)9H and T(7;18)50H (Tab. 2) tend to confirm those for T(7;?)2Sn in suggesting that there is noncomplementation, both with maternal duplication of part of chromosome 7 combined with paternal deficiency, and also with maternal deficiency combined with paternal duplication. We can be confident that the results with T9H are connected with chromosome 7 rather than chromosome 15 because there is other evidence of normal complementation for chromosome 15 duplications and deficiencies with reciprocal crosses of Robertsonians involving this chromosome (3,22). There is also evidence for normal complementation of the distal part of chromosome 18 (36).

If the data in Tab. 2 are combined with knowledge of the breakpoints in the translocations concerned (35), then the regions involved in the noncomplementation phenomena can be delineated (Fig. 2). In this analysis, T2Sn and T9H nil results for proximal markers in males have been disregarded because of small numbers of progeny. Results suggest that the regions involved in the 2 types of noncomplementation are different (4). The region associated with maternal duplication combined with paternal deficiency lies between the breakpoints of T2Sn and T50H, i.e., within about 10 crossover units distal to the *c* locus and probably including the *Hbb* locus. The region associated with paternal duplication combined with maternal deficiency must be distal to the T50H breakpoint (because of apparent normal complementation with T50H+ *pp* males), and thus distal to the *Hbb* locus.

Chromosome 8

The results obtained with T(2;8)26H (Tab. 1) suggested that chromosome 8 might be implicated in noncomplementation as well as chromosome 2, so heterozygotes for another translocation, T(8;16)17H, were intercrossed, with use of yellow (*e*) as a distal marker on this chromosome. When males

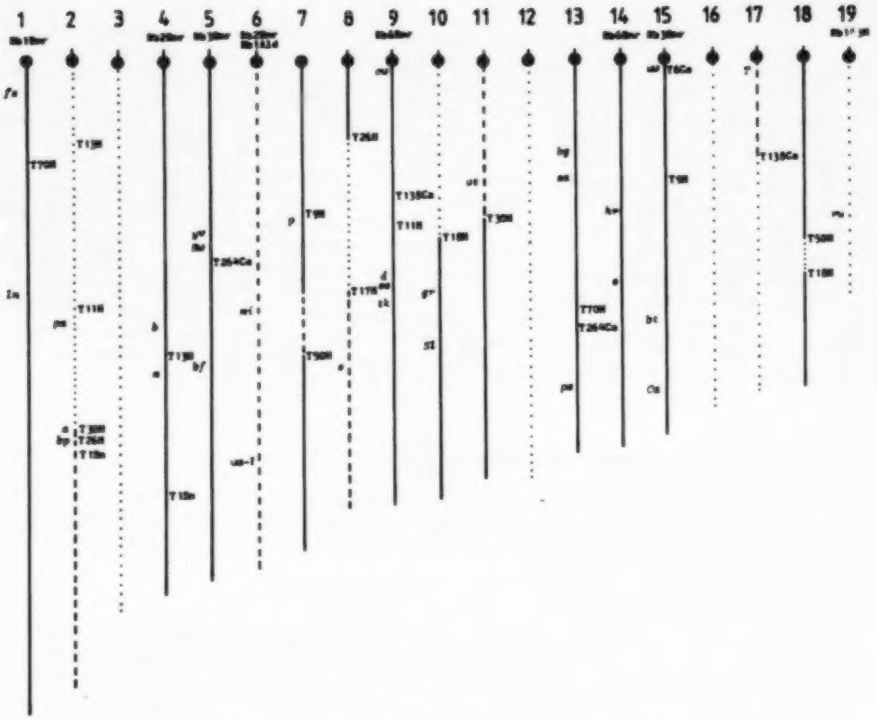

Fig. 2. Diagram of mouse autosomes to show areas in which complementation involving a maternal duplication with a paternal deficiency is normal (solid line), defective (broken line), or in which conclusive evidence is lacking (dotted line). Breakpoints are shown of those reciprocal translocations used in these studies, while names of Robertsonian translocations used are placed above the chromosome concerned.

were ee there was normal complementation, but when females were ee no yellows were found in 30 offspring (36). Cattanach et al. (9) have also found normal complementation with paternal disomy for chromosome 8 combined with maternal nullisomy in Rb(8.12)5Bnr/+ intercrosses.

Chromosome 11

Cattanach and Kirk (8) have intercrossed heterozygotes for Rb(11.13)-4Bnr in which chromosome 11 is marked with vt and chromosome 13 with sa. They discovered that when the mother was homozygous for vestigial-tail (vt) and the father was wild-type, any vt offspring were only half the normal size at birth, but with the reciprocal cross they were larger than normal by a factor of about 1.5. Further work with T(2;11)30H has confirmed this finding and has shown that it is associated with the proximal part of chromosome 11. Clearly this is a phenomenon similar to those described earlier, but one in which both maternal and paternal disomies (with corresponding nullisomies) are having an effect.

Chromosome 17

Johnson (19,20) studied the hairpin-tail mutation, T^{hp}, which is now known to be a small deficiency on chromosome 17. He reported that it was viable in the heterozygote when derived from the father, but lethal when derived from the mother, an effect which is definitely nuclear rather than cytoplasmic (27). A T-associated maternal-effect locus (Tme) on chromosome 17 has now been described and mapped (42). Here we are dealing with a lethal effect of maternal deficiency for a certain region rather than a failure of complementation. However, Lyon and Glenister (23) intercrossed heterozygotes for T(9;17)138Ca in which the proximal region of chromosome 17 was marked with Tt. They found higher frequencies of successful complementation when genetically marked maternal gametes with a duplication of the proximal region of chromosome 17 met paternal ones with a deficiency, than in the reciprocal cross. They concluded that this was probably another example of the phenomenon described by Johnson, since maternal deficiency for a proximal region in chromosome 17 was implicated in both cases.

EVIDENCE FOR SUCCESSFUL COMPLEMENTATION

This is best dealt with in 2 sections, one concerned with whole chromosomes, studied through the use of genetically marked Robertsonian translocations, and the other concerned with reciprocal translocations.

Robertsonian Translocations

Lyon (22) has recently reviewed her own and Cattanach's studies on nondisjunction in genetically marked Robertsonian heterozygotes (Tab. 3). In 6 chromosomes (numbers 1, 4, 5, 9, 14, and 15), disomic gametes of either sex are known to complement successfully the corresponding nullisomic gametes. Findings for chromosome 1 have been confirmed (10) on the compound Rb(1.3)1Bnr/Rb(1.15)2Ct, while those for chromosome 15 are in line with those reported for the compound Rb(4.15)4Rma/Rb6.15)1Ald (3). The negative results for chromosome 19 shown in Tab. 3 have been put in parentheses because the nondisjunction rate in the laboratory-derived Rb163H heterozygote is very low, so that the absence of marked young in 5,260 examined is consistent with normal complementation (25).

Tab. 3. Evidence from genetically marked single Robertsonian translocations on complementation of disomic and nullisomic gametes. See Ref. 22 and 25.

Chromosome	Markers	Translocation	Successful complementation with maternal Nullisomy	Successful complementation with maternal Disomy
1	ln	Rb(1.3)1Bnr	Yes	Yes
4	b, m	Rb(4.6)2Bnr	Yes	Yes
5	bf	Rb(5.15)3Bnr	Yes	Yes
6	wa-1	Rb(4.6)2Bnr	Yes	No
8	e	Rb(8.12)5Bnr	Yes	--
9	d, se	Rb(9.14)6Bnr	Yes	Yes
13	pe	Rb(11.13)4Bnr	Yes	--
14	hr, s	Rb(9.14)6Bnr	Yes	Yes
15	bt	Rb(5.15)3Bnr	Yes	Yes
19	ru	Rb(9.19)163H	(No)	(No)

Reciprocal Translocations

Currently available data (Tab. 4) are derived from a number of sources (quoted) and are in good agreement with results from Robertsonian intercrosses, as well as giving some additional data. Although information has only been given here under the chromosome to which the genetic marker belongs, it should be remembered that a maternal duplication and paternal deficiency for a proximal or distal region on one of the chromosomes concerned will necessarily be accompanied by a maternal deficiency and paternal duplication for the corresponding region on the other chromosome. Thus Tab. 4 contains much more information than would appear at first sight. On the whole, the data reinforce the results from intercrosses with Robertsonian translocations given in Tab. 3, but there is additional information for chromosomes 10 and 18, showing successful complementation distal to the T18H breakpoints on these chromosomes, and for chromosome 17.

DISCUSSION

Figures 2 and 3 summarize the present situation with regard to complementation of unbalanced genomes, as obtained from translocation intercrosses with genetic markers. Defective complementation has been found associated with chromosomes 2, 6, 7, 8, and 11, with aberrant behavior also in 17. In all these chromosomes difficulties arise with maternal duplication of a chromosome or chromosome region combined with a paternal deficiency (Fig. 2). In chromosome 7, however, paternal duplication of a separate region, combined with maternal deficiency, seems to cause trouble, as also in chromosome 11. Both sorts of duplication and deficiency complement each other to give fully normal zygotes when chromosomes 1, 4, 5, 9, 13, 14, and 15 are involved. The same is probably true for chromosome 10, but information on the others (3, 12, 16, 18, and 19) is incomplete or absent altogether. As far as the X chromosome is concerned there is plenty of evidence for survival of XOs after maternal or paternal X-chromosome deficiency, and also for survival of an XXY in which both X chromosomes come from the mother (30). However, no mouse with the XXX karyotype seems to have been discovered yet, although human XXXs are well known.

Only with respect to chromosomes 2 and 8 (where it leads to perinatal death) and chromosome 11 (where it affects size but not survival) is anything definite known about the time of action of the complementation defect, although it is clearly pre- or neonatal in chromosomes 6 and 7. For further investigation, good genetic markers with early expression are needed. Several such are available on chromosome 7 (e.g., c and p), so

Tab. 4. Evidence for normal complementation of unbalanced gametes from genetically marked reciprocal translocations.

Chromosome	Translocation	Marker Proximal	Marker Distal	Complementation with maternal Duplication	Complementation with maternal Deficiency	Reference number
1	T(1;13)70H	fz	ln	Yes	Yes (fz)	38 and unpubl.
5	T(5;13)264Ca		bf	Yes	Yes	36
9	T(9;17)138Ca	cw		Yes	Yes	23
10	T(10;18)18H		gr, Sl^{con}	Yes	Yes	36
13	T(5;13)264Ca	sa	pe	Yes	--	38
14	T(14;15)6Ca	hr		--	Yes	12
17	T(9;17)138Ca	T, t		Yes	Yes*	23

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* Reduced numbers.

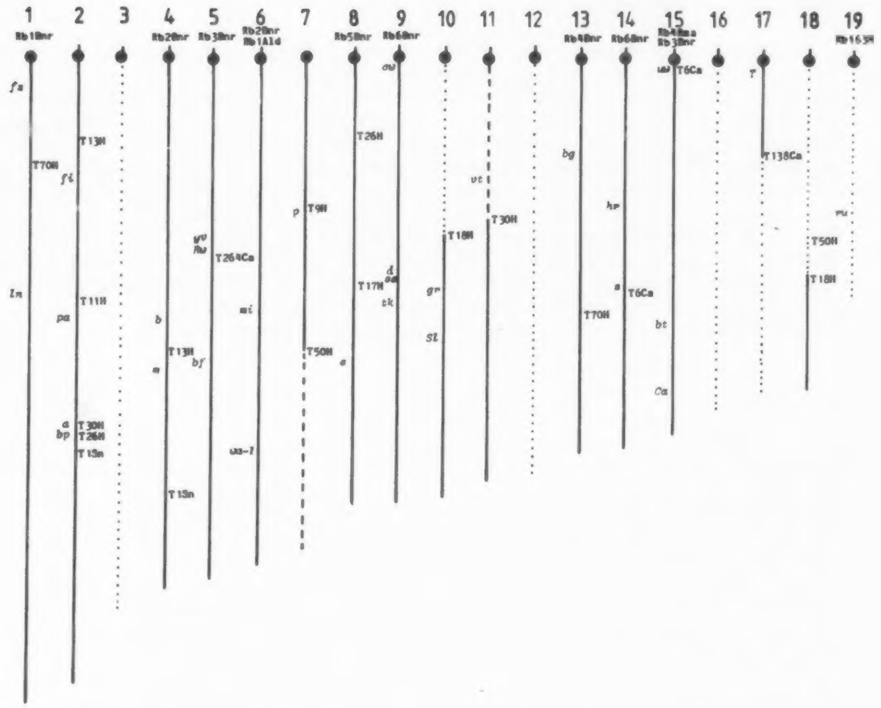
1
Rb1Bnr
2
3
4
Rb2Bnr
5
Rb3Bnr
6
Rb2Bnr
Rb1Ald
7
8
Rb5Bnr
9
Rb6Bnr
10
11
12
13
Rb4Bnr
14
Rb6Bnr
15
Rb4Bnr
Rb3Bnr
16
17
18
19
Rb163H
fs
T70H
ln
T13H
fi
pa
T11H
a
bp
T30H
T26H
T13n
b
T13H
m
T13n
vv
Rw
bf
T264Ca
mi
wa-1
p
T9H
T50H
T26H
T17H
e
ow
d
se
tk
T18H
gr
Sl
vt
T30H
bg
T70H
hr
s
T6Ca
wa
T6Ca
bt
Ca
T
T138Ca
T50H
T18H
ru

Fig. 3. Diagram of mouse autosomes to show areas in which complementation involving a paternal duplication with a maternal deficiency is normal (solid line), defective (broken line), or in which conclusive evidence is lacking (dotted line). Symbols as in Fig. 2.

this has been selected for special attention in the future. The fact that diploid parthenogenones (with maternal duplication and paternal deficiency of all chromosomes) can survive up to, but no further than, the 25-somite or 11½-day stage (21,26,40) suggests that none of the complementation defects will be lethal very early in embryonic life.

Further light is shed on the origins of defective complementation by the findings of Surani et al. (41) that when a male pronucleus is added to haploid parthenogenetic eggs, many successfully develop to term, but when a female pronucleus is added none do so. Although most of the latter group implanted, they developed very poorly thereafter, with only the most advanced reaching the 25-somite stage, as with diploid parthenogenones. The authors suggest that this developmental failure is connected with a lack of paternal imprinting of the genome during gametogenesis in the absence of a paternal set of chromosomes, which might lead to delayed expression of some paternal genes. Further work by the same authors (1) has shown that eggs with 2 male pronuclei also fail to develop to term and are very retarded, even though the trophoblast develops better than in those with only female pronuclei. McGrath and Solter (27) have also reported a failure of androgenetic or gynogenetic embryos to come to term.

These findings are what would be expected from the defective complementation found with a number of chromosomes when there is maternal duplication and paternal deficiency, and with part of chromosome 7 when there is the reverse. Work on t complex deletions (19,20,42) and on the T138Ca translocation (23) suggests that deficiency of a specific locus (Tme) on chromosome 17 in the maternal gamete can also have adverse effects. Specific imprinting of both maternal and paternal genomes may occur during gametogenesis, so that both are required in the zygote for full-term development (41). However, Lyon and Rastan (24) considered that imprinting should be regarded as "a phenomenon conferred on the maternal chromosomes in the oocyte," which might affect the timing of gene action in development.

Thus, the possibility remains that duplication of parental genomes per se might have a harmful effect on development, independent of that generated by the resultant trisomy or triploidy. If so, the severity of effect in certain trisomics or triploids might depend partly on parental derivation of the extra chromosome(s). It may be relevant that in a survey of triploids (18), all 51 that were paternally derived were partial hydatidiform moles, while only 3 of 15 maternally derived ones were of this type. Hassold et al. (17) studied the parental origin of human autosomal trisomies and found that most were derived from primary nondisjunction in the female (see also Ref. 16). However, trisomy 21 was more likely to be paternally derived than any of the other autosomal trisomies for which data were available. The authors considered whether this might be connected with differences in selection against trisomies of maternal and paternal origin, but concluded that probably this was not so. However, the amount of information on the parental origin of trisomies other than 21 is still very limited and confined to chromosomes 3, 4, 9, 13, 14, 15, 16, and 22 (16).

The rapidly increasing body of knowledge on chromosome regions that are homologous in man and mouse (7,29) can give some pointers to which human chromosomes may have regions homologous to those which lead to defective complementation in the mouse. Thus, the distal part of mouse chromosome 2 has homologies with human chromosome 20q; main homologies for mouse chromosome 6 seem to be with human 12p, but human chromosomes 2 and 7 are also involved. The region around the Hbb locus on mouse chromosome 7 is homologous with human 11p, while regions of interest on mouse chromosomes 8, 11, and 17 show homologies with human chromosomes 16q, 17, and 6p, respectively. Of all these human chromosomes only 16 has been studied at all with respect to the parental origin of the trisomy; much more work will be needed before the position is clear.

These findings have some important implications for those tests for nondisjunction that use genetic markers and rely on complementation of duplications and deficiencies to allow the marker phenotype to appear (10,25,37). It is clear that at least for chromosomes 2, 6, 7, and 8, tests for nondisjunction or chromosome loss in male germ cells, by crossing treated wild-type males to female Robertsonian heterozygotes that are homozygous for genetic markers on any of these chromosomes, will give negative results. The same is true for similar tests on female germ cells if a male Robertsonian heterozygote was used with a genetic marker on chromosome 7, unless, perhaps, there is prenatal diagnosis with a suitable embryonic marker like c (albinism).

Much more work is needed before it will become clear whether any other chromosomes are implicated in this noncomplementation phenomenon. In some chromosomes (e.g., 12) there is a lack of suitable markers, while in others the right translocations are lacking, since male-sterile ones are clearly useless for this work. Male sterility is also a frequent problem when using compounds of 2 Robertsonians with monobrachial homology (13), although from other points of view these would seem ideal since they show remarkably high frequencies of nondisjunction (15). Only by a combination of different methods can we hope to elucidate the extent of this phenomenon over the whole genome.

ACKNOWLEDGEMENTS

We are indebted to Dr. Mary F. Lyon for her critical comments. This paper is dedicated to Dr. George D. Snell, in recognition of his pioneering studies on complementation phenomena more than 40 years ago, and of his superlative record of research in mouse genetics for over 50 years.

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Note Added in Proof: Cattanach and Kirk (Nature 315:496-498, 1985) have now shown that in T(2:11)30H/+ intercrosses, maternal duplication and paternal deficiency for the distal part of chromosome 2 leads to neonatal lethals with long flat-sided bodies, while the reciprocal condition leads to postnatal lethals with short square bodies and hyperkinetic behavior. Thus paternal duplication/maternal deficiency has an effect in chromosome 2 as well as chromosomes 7 and 11.

DISCUSSION

EVANS: Are you implying, by extrapolation from your findings in the mouse, that the consequences of nondisjunction in man might be influenced by the sex in which it occurred? If so, is there any evidence for this?

SEARLE: Yes, this is what I suggest. I do not know of any confirmatory evidence, but few human chromosomes have been studied sufficiently to reveal this.

EVANS: What are the human homologies for the segments of mouse chromosome 7 that you think are involved in this phenomenon?

SEARLE: That associated with maternal duplication has homologies with human 11p, but no homologies have been found yet for the more distal segment.

EXPERIMENTAL APPROACHES FOR THE DETECTION OF CHROMOSOMAL MALSEGREGATION OCCURRING IN THE GERMLINE OF MAMMALS

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ABSTRACT

Existing and newly proposed methods to detect the induction of heritable aneuploidy are summarized, and their favorable and unfavorable features discussed. Among the tests involving direct chromosomal examination, those involving study of pronuclear chromosomes at first cleavage are judged to be the most universally informative and reliable, provided tertiary trisomy can be ruled out. Measurement of postmidterm (fetal) death is proposed as a trisomy prescreen that can be readily combined with a dominant-lethal test. Among the genetic procedures for identifying the results of malsegregation events, direct detection of aneuploids has a number of advantages over complementation methods, in which only a fraction of the products of aneuploid gametes is detectable. Direct detection of aneuploids must, however, be restricted to sex chromosomes if postnatal animals are examined. A genetic marker system to detect autosomal trisomies in fetuses is proposed. An examination of experimental parameters that might maximize induction of malsegregation leads to the recommendation to include preleptotene among exposed germ-cell stages.

INTRODUCTION

The process of malsegregation of chromosomes is of greatest concern to man when it results in whole-body aneuploidy, especially trisomy. Because of this, many of the questions asked about induced nondisjunction, both in regard to basic mechanisms involved and in regard to methodologies used for the detection of induced malsegregation, must be focused on the germline.

The mammalian germline contains mitotic, meiotic, and postmeiotic cells. Therefore, processes and structures additional to spindle fibers and kinetochores play a role in normal segregation and may be vulnerable to physical and chemical agents. Results of studies with somatic cells are thus only partly applicable to the germline; within the germline, each individual cell type may present specialized conditions for induction of events that eventually lead to malsegregation.

A number of prodcedures have been used to detect and measure the induction of heritable aneuploidy in experimental mammals. Others have been proposed but not yet tried. Both groups of methods will be briefly described and their favorable and unfavorable features will be examined.

COMPONENTS OF GERMLINE NONDISJUNCTION TESTS

The emphasis in studies on the mammalian germline has been on the detection of trisomy, and thus on agents that induce trisomy. Reasons for the relative neglect of the (usually complementary) monosomic types are that (a) in cytogenetic studies of germ cells and zygotes, real and artifactual chromosome losses cannot be distinguished from each other; and (b) in studies involving later stages, the monosomics (except for XO) are missing as a result of their early embryonic death. Autosomal loss events can, however, be detected by genetic complementation.

In developing methods to determine whether an agent is capable of inducing chromosome malsegregation in the germline, the following test parameters must be considered: cell type exposed, developmental stage examined for response, type of analysis carried out, and signal of malsegregation. Other variables, such as strain and age, will be examined later (see pp. 391-392).

Cell Type Exposed

There is no a priori reason to exclude any germ-cell type from potential vulnerability to damage that can result in an aneuploid conceptus. Mitotic nondisjunction induced in gonial divisions would eventually lead to disomic gametes, and its induction has been claimed (39). The meiotic chromosome misbehaviors that can, at least theoretically, produce aneuploid conceptuses include reduced recombination (Grell, this Volume), pairing failure (nonconjunction), nondisjunction at the first or second meiotic division, precocious centromere division in meiosis 1, and extra replication of a chromosome (3). While meiotic segregation errors can obviously not be induced by treatment of postmeiotic germ cells, it is at least theoretically possible that some form of damage could be inflicted that would later on cause nondisjunction at first cleavage.

The accessibility of the various stages is very different in the two sexes and changes with age. In adult males, where stem cells are being constantly replenished, the treatment-to-conception interval provides an accurate identification of the treated germ-cell stage that entered into the conception (26). In adult females, neither premeiotic nor postmeiotic germ-cell stages are available for treatment, and all oocytes are in a modified diplotene stage, except at proestrus and estrus when a limited number progress to metaphase 2.

Developmental Stage Examined for Response

Indicators of the potential generation of aneuploid conceptuses have been looked for in parental germ cells, specifically in the first and second meiotic divisions and in spermatids or spermatozoa. Since the maternal and paternal chromosome complements remain separate in the two pronuclei of the zygote, the first cleavage division provides an extension of germ-cell analysis. Subsequently, embryos (at various stages of development), fetuses, and postnatal animals can all provide evidence that malsegregation of parental chromosomes has occurred.

A systematic study of the phenotypes of individual trisomies in the mouse has been underway in recent years (12,13). Tools for this study are combinations of Robertsonian translocations with monobrachial homology for the chromosome for which trisomy (resulting from frequent spontaneous nondisjunction) is to be studied. This work indicates that probably no autosomal trisomy (Ts) survives birth, except for Ts19 which may live a few days (12,57) (Tab. 1). Postnatal observations must therefore be restricted

Tab. 1. Survival of aneuploidies in the mouse.

Latest survival[a]	Monosomies	Trisomies	Phenotype[d]
Day 3-9 pc[b]	MS 1-19		
Day 10-13 pc		Ts 2-5, 7-9, 11, 15, 17	
Day 14-15 pc		Ts 1, 6, 10	Ts 1: cyclopia, etc.
Day 16-18 pc		Ts 12, 13, 14	Ts 12: exencephaly, microphtalemia Ts 13: cleft palate
Term		Ts 16, 18, 19	
Postnatal	XO	XXY, XYY, XXX ?, (Ts 19)[c]	

[a] Based on reports of Gropp and coworkers (12,13). Survival time is strain-dependent.

[b] pc = postconception. By Gropp's designation, the day of the vaginal plug = day 1 pc.

[c] Longest documented survival: day 13 after birth. Most die around the time of birth.

[d] Only regularly found phenotypes are shown. In addition, exencephaly occurs sometimes in Ts 14; cleft palate occurs frequently in Ts 18, and sometimes in Ts 19; and ventricular septal defects are often found in Ts 10, 12, 14, 16, and 19.

to sex-chromosome trisomics or to animals produced by the complementation of hyperhaploid and corresponding hypohaploid gametes.

Type of Analysis and Signal of Malsegregation

The two major groups of approaches to diagnosing malsegregation are direct chromosomal examination and genetic detection systems. Within each of these broad categories, a variety of techniques have been used or proposed, and these will be discussed in the context of the individual methods. Signals include asynaptic axes, hyperhaploid complements, supernumerary sex bodies, fetal death, diagnostic combinations of genetic markers, and others.

TEST SYSTEMS INVOLVING DIRECT CHROMOSOMAL EXAMINATION

Observations Involving the First Meiotic Prophase

Apart from the chance of detecting the results of gonial nondisjunction, endpoints scored at this stage are not in themselves the result of malsegregation, but may be predictive of the imminence of this event. Among indicators that the pairing of homologs is less than optimum are synaptonemal complex anomalies seen in microspread preparations (6; Moses et al., this Volume), or lowered chiasma frequency and/or occurrence of univalents at diakinesis-metaphase 1 (e.g., Ref. 18, 30, 43, and 54). It has now been shown by a number of investigators (19a,30,43,46) that the univalents seen at the first meiotic division do not necessarily represent aneuploids scored at metaphase 2. Many such univalents may be artifacts of slide preparation (30). Furthermore, true univalents may be normally segregated after entering the distributive pool (11). It has also been suggested that gametes inheriting an unpaired chromosome may not complete development (24).

Metaphase-2 Counts

Analysis of the second meiotic metaphase would appear to be the most direct way of detecting any malsegregation that had occurred in (or had been passed down through) the first meiotic division. Consequently, many investigators interested in possible induction of nondisjunction have counted elements (normally paired chromatids) in M2 (for references, see Ref. 3, 16 and 35). Secondary spermatocytes are generally obtained from testicular suspensions, while secondary oocytes are obtained either from the oviduct after ovulation or following in vitro culture of dictyate primary oocytes removed from the ovary. The use of C-banding methods for centromere regions (29) has enhanced the accuracy of chromosome counts which otherwise can be unreliable because of the special morphology of metaphase-2 chromosomes.

Indeed, aneuploidy estimates based on M2 observations are considerably higher than those obtained by other means. One example comes from irradiated female mice. On the assumption that the frequency of hyperhaploid M2s scored cytogenetically by these investigators was equivalent to that of hypohaploid secondary oocytes, each of which should result in an early-dying conceptus, one may derive a minimum estimate for expected dominant-lethal frequency (1). Dominant-lethal experiments on irradiated females, however, have yielded much lower deficits of living embryos (1). Similarly, the incidence of hyperhaploidy found in pronuclei by Hansmann (15) was considerably below that diagnosed in M2 by the same investigator.

Metaphase-2 analysis has provided evidence that precocious centromere division in meiosis 1 can occur spontaneously (17,30), but induction of this event has not been recorded.

The main difficulty with the M2 method in the mouse is that, except for the sex chromosomes, individual chromosomes are not identified, let alone analyzed for rearrangements (as would be possible with banded chromosomes). For this reason, it is possible that extra elements found in M2 might result not from a primary malsegregation event but secondarily from a rearrangement, e.g., by 3-1 segregation of a reciprocal translocation induced in an earlier germ-cell stage. In Drosophila, Parker and Williamson (28) have demonstrated the generation of aneuploidy from half-translocations induced in oocytes by radiation. Extra elements in M2 could thus be the result of clastogenic, rather than malsegregation, events (35). Examples of such occurrences are reported elsewhere (19a,51).

Analysis of Postmeiotic Germ Cells

Postmeiotic germ cells exist only in the male. The round spermatids of Microtus oeconomus, the northern vole, normally contain one dark-staining X or Y body. The presence of 2 dark-staining bodies in such cells has been taken as evidence for sex-chromosome nondisjunction (49). Results from a similar system, the scoring of fluorescent double bodies in human sperm, have been seriously questioned because they are not supported by DNA measurements (47) or by cytogenetic data obtained in parallel with fluorescent-body counts (58).

Analysis of Pronuclear Chromosomes at First Cleavage

The paternal and maternal chromosome contributions remain separate in the pronuclei of the zygote and can be studied in the prometaphase of the first cleavage division (e.g., Ref. 7, 8, 19, 22, 50, and 55). The two chromosome sets (which remain distinct if syngamy is prevented) can often be identified as to their parental source on the basis of condensation (maternal set more condensed), and can be unequivocally identified if one parent carries a marker chromosome, such as T6 (22).

First-cleavage examination is the earliest analysis involving the conceptus and reveals the result of nondisjunction at any germ-cell stage. Chromosome morphology in the zygote makes reliable counting easier than that at M2. The complement contributed by the untreated parent provides an internal control. Unless this can be ruled out by chromosome banding, some of the hyperhaploidies seen in first-cleavage studies, like those in M2 analyses, could be the result of tertiary trisomy, rather than being indicators of primary malsegregation events.

Cytogenetic Analysis of Embryos

Analysis for aneuploidy has been carried out in preimplantation mouse embryos and in postimplantation stages ranging from days 6-14 postconception (pc). Embryonic stages of other mammalian species have also been studied (3).

The systematic use of Robertsonian translocations to study the phenotype of mouse trisomics (see pp. 378-379) has revealed that while most trisomies survive to day 9 of gestation (by Gropp's terminology, vaginal plug = day 1), about half of these are dead 3 days later. Therefore, studies extending past day 10 (as did several of those summarized in Ref. 3) deal with selected populations. At the other end of the spectrum, use of very early embryonic stages decreases the chance of finding a sufficient number of metaphase cells to draw conclusions about individual embryos.

Analysis of embryos multiplies the cytogenetic effort required in that several cells are scored to assess the chromosome complement of a single conceptus. This multiple scoring, on the other hand, increases the accuracy of the observation, especially since it can be combined with banding analyses, and allows the detection of mosaicism. In the case of 2n+1/2n mosaics, it may be impossible to determine whether the animal started life as a whole-body mosaic and lost the supernumerary chromosome from some cell lineages, or whether the conceptus was originally normal and the mosaicism resulted from a mitotic event during embryogeny. The favorable features and drawbacks of test systems involving direct chromosomal examination are summarized in Tab. 2.

TRISOMY SCREENS COMBINABLE WITH OTHER ASSAYS

Postmidterm Death: A Proposed Screen

The outcomes of meiotic nondisjunctions are presumably equal numbers of monosomic and trisomic conceptuses. Those in the former class will die before or shortly after implantation (12). They are thus automatically included in the scores from standard dominant-lethal experiments, along with conceptuses whose chromosome imbalance results from clastogenic events. As noted (see pp. 378-379 and section above), it has recently become apparent that single trisomies for most chromosomes are lethal considerably later, several of them past the stage (days 12-14 pc) at which dominant-lethal scoring is often done. At our laboratory, we are therefore exploring the feasibility of using postmidterm death (occurring days 11-17 pc) as a prescreen for the induction of trisomy.

Tab. 2. Test systems involving direct chromosomal examination to detect malsegregation (MS).

Stage analyzed	MS signal	Favorable features of test	Drawbacks
Primary spermatocyte (zygotene, pachytene, diakinesis)	Synaptonemal complex anomalies Low chiasma frequency Univalents	Synaptic errors detectable. Prior gonial nondisjunction detectable. Cell availability good in male.	Univalents can be artifacts. True univalents (nonsynapsis) may not lead to distribution errors.
M2 (male or female)	Hyperhaploidy	Can distinguish nondisjunction from precocious centromere division. Cell availability good in males. C-banding improves scoring.	Supernumerary elements could result from clastogenic events. Dyad counts may be equivocal (especially without C-banding). Hyperhaploid frequency exceeds MS estimates derived by other means. M2 malsegregation not detected.
Spermatid or spermatozoa	Supernumerary darkstaining (or fluorescent)body	Ease of scoring. Cell availability excellent. M1 and M2 events detectable.	Supernumerary element(s) could result from clastogenic action, etc. Results not substantiated by DNA measurements or cytogenetic data.
Zygote	Hyperhaploidy in paternal or maternal chromosome set	Detects MS in any germ cell stage. Identifies parental source of MS. Chromosome set from untreated parent is internal control.	Supernumerary element(s) could result from clastogenic action (can be ruled out by banding). Cell availability somewhat limited.
Early through midgestation embryo	Trisomy	Chromosome banding possible. Several cells per individual: greater certainty.	Laborious. Selection against aneuploids with advancing age of conceptus. Possible confusion with MS during embryogeny.

To date, 2 suspected trisomy-inducers have been used in this endeavor. Ethyl alcohol, administered at about the time of sperm entry (M2 of the oocyte), was reported by Kaufman (19) to produce hyperhaploid maternal complements at first cleavage division. A comparable treatment was administered by Generoso's group (55), and pregnant females sacrificed 17 days after mating. Uterine contents were classified as surviving fetuses, and implants dead before day 11 pc (early deaths [EDs]), or dead days 11-17 pc (late deaths [LDs]) (Tab. 3). The frequency of EDs was not significantly increased by the treatment. While monosomies, if induced, would be part of the ED class, treatment-unrelated EDs are so common (approx. 5%) as to obscure any small incidences of induced EDs. The late-death (LD) frequency, on the other hand, was significantly increased in the treated group, the excess being about 1.3%. In a parallel but smaller series, the investigators found the incidence of hyperploid first cleavage metaphases to be also increased, the excess over controls being 2.9%.

Another test of the utility of scoring postmidterm death is being carried out with 6-mercaptopurine, a chemical which in earlier studies (10) had been shown to yield XXY and XYY individuals among offspring of males treated 31.5-35.5 days earlier. We have, in fact, found the incidence of LDs to be higher in the treated groups than in controls, but the difference is not yet statistically significant.

The postmidterm-death prescreen has the attraction of being attainable by a simple modification of the usual procedure for the dominant-lethal test (DLT), which is often the only whole-mammal assay included in the exploration of mutagenic effects of a chemical. The modification does not detract from the normal capabilities of the DLT. With the typical DLT sample sizes, excess LD frequencies of >3% would probably be detectable. To determine whether these, in fact, represent autosomal trisomies would require follow-up with a specific aneuploidy test.

Trisomy Information from Routine Heritable-Translocation Tests

In the heritable-translocation test (9), adult sons of treated and control animals are tested for "fertility." Follow-up on the fully-sterile class of sons sometimes reveals these to be XXY or XYY. Information on possible induction of sex-chromosome trisomy is thus obtained, without extra effort, as an ancillary result of the heritable-translocation assay. Trisomy prescreens that are combinable with other assays are summarized in Tab. 4.

Tab. 3. Results of uterine analysis on day 17 postconception. Data from Washington et al. (55).

Treatment	Number of implants[a]				
	Alive (SF)	Dead before day 11 pc (ED)	Dead day 11-17 (LD)	$\frac{ED}{SF+ED+LD}$	$\frac{LD}{SF+ED+LD}$
Ethanol	1,085	76	21	0.064	0.018[b]
Control	1,003	56	5	0.052	0.005

[a] SF = surviving fetuses; ED = early deaths; LD = late deaths; pc = postconception.
[b] P = 0.002

Tab. 4. Trisomy prescreens combinable with other assays.

Stage analyzed	Trisomy signal	Favorable features of test	Drawbacks
Day 12-19 fetus	Death after day 10 pc	Can be "piggy-backed" on dominant-lethal test with no extra effort.	Is a prescreen, sometimes requiring follow-up. Requires trisomy frequencies >3% for easy detection.
Adult offspring	Male sterility due to sex-chromosome trisomy	Results obtainable during routine heritable-translocation study (no extra effort needed).	Most of F_1 male sterility is due to translocation (method justifiable only as adjunct to translocation test).

GENETIC METHODS FOR DETECTION OF MALSEGREGATION

Since monosomy or trisomy for any mouse autosome is lethal by the time of birth or shortly thereafter, aneuploidy-detection methods that depend on postnatally expressed genetic markers have been of 2 types: (a) they have employed complementation to achieve chromosomally balanced survivors of autosomal loss/gain events; or (b) they have utilized numerical sex-chromosome anomalies which are viable. Direct genetic detection may also be achievable for certain autosomal aneuploidies by using markers expressed in fetuses, a method proposed below (see pp. 389-391). Genetic methods are summarized in Tab. 5.

Complementation Methods

Almost 40 years ago, Snell (42) showed that complementary unbalanced gametes from intercrosses of mice heterozygous for reciprocal translocations could produce viable offspring that were identifiable by a recessive marker. Complementation from other types of parent stocks is now being utilized to detect occurrence of nondisjunction. A generalized scheme is shown in Fig. 1. The untreated (tester-stock) mate used in the cross introduces a relatively high proportion of unbalanced (nullisomic or disomic) gametes that can complement the opposite type of unbalanced gamete produced by the treated (or control) animal. With the optimum use of genetic markers, one can distinguish the two types of complementation-derived offspring from each other (nullisomic gamete from treated parent combining with disomic gamete from tester stock, and vice versa), and both types from the offspring derived from two normal gametes.

Robertsonian tester stocks. Frequent nondisjunction, the prerequisite for a good tester stock, is observed in mice heterozygous for certain Robertsonian (Rb) translocations, particularly those derived from *Mus poschiavinus*. Gropp and Winking (14) found the nondisjunction rates for different Rbs to range from 2 to 28% in male carriers; rates were even higher in females. Intercrosses of Rb translocation heterozygotes were used by Lyon et al. (20) to measure the frequency of spontaneous first-division nondisjunction in Rb stocks. For various reasons, such intercrosses are, however, not useful for tests of nondisjunction induction. Subsequently, Searle and Beechey (41) described a number of genetic schemes in which chromosomally normal mice would be treated with a test agent and mated to tester stocks carrying single or multiple Rbs. One combination involved two RBs with monobrachial homology, a configuration found by Gropp et al. (13) to produce particularly frequent nondisjunction for the common arm.

Tab. 5. Genetic methods for the detection of malsegregation (MS).

Method[a]	Favorable features of test	Drawbacks
Complementation: tester stock with frequent MS: general features	Scoring easy (external phenotype).	Only fraction of products of aneuploid gametes detectable. For some chromosomes, duplicate set derived from female parent may be lethal.
1. Robertsonian (Rb) testers[b]	High MS frequencies for chromosomes involved in Rb(s).	In several Rb combinations, males are sterile. Low littersize due to death of aneuploid offspring.
2. Heteromorphic bivalents testers[c]	Increased MS for entire genome.	Some combinations may be male-sterile or male-lethal.
Direct detection of aneuploids		
1. Numerical sex-chromosome anomaly (NSA) test	Scoring easy (external phenotype). Spontaneous hyperploids rare (<0.04%). Parental source of aneuploidy identifiable. Little embryonic selection against hyperploids. Hypoploid type is not an artifact (but may not result from MS).	Detects <50% of M2 events. Mechanisms governing X-Y segregation may differ from those governing autosomal segregation.
2. Trisomy-marker test: linked recessives in repulsion[c]	All products of gametes disomic for marked chromosome(s) are recognizable. No restriction as to sex of treated parent. No selection due to markers. Can be combined with dominant-lethal test or postmidterm-death prescreen for trisomics.	Requires examination of fetuses. For some chromosomes, duplicate set derived from female parent may be lethal.

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[a] Exceptional offspring resulting from nondisjunctional events are initially identified by genetic markers. Cytogenetic verification may follow.

[b] Stock heterozygous for a single Rb or for combinations of Rbs.

[c] Proposed. Exploratory work needed to show feasibility.

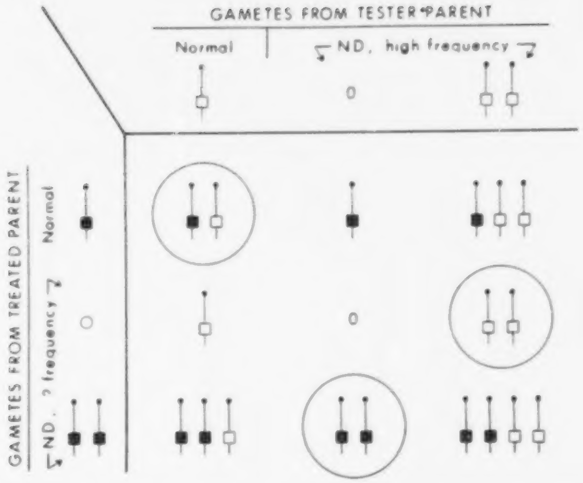

Fig. 1. Generalized scheme for the use of genetic complementation methods to detect induced and spontaneous nondisjunction. Chromosomal contributions by the treated (or control) parent are shown in the vertical margin and those by the tester-stock parent in the horizontal margin. Only one chromosome, genetically marked (black or white box), is represented. As a result of normal segregation, each parent contributes a single dose of this chromosome; nondisjunction causes the transmission of zero or 2 doses. The body of the matrix shows the zygotic constitution with respect to the marked chromosome. Viable (disomic) conceptuses are circled. The markers are chosen so that the normal, heterozygous type (one black and one white box) is distinguishable by external appearance from each of the complementation types derived from nondisjunction in both parents (2 black boxes or 2 white boxes). (Markers used can be codominants at one locus, or recessives at two linked loci). The tester stock is chosen so as to provide a high frequency of nullisomic and disomic gametes, optimizing opportunities for complementation of disomic or nullisomic gametes, respectively, produced by the treated (or control) parent. Various high-nondisjunction tester stocks have been used or proposed (see text).

Some of the methods described by Searle and Beechey (41) have recently been tested in X-ray experiments by Cattanach et al. (5). The untreated tester stock in one set of experiments was heterozygous for a single Rb marked in one arm. Thus, induced nondisjunction for only one chromosome (No. 1) was studied. In another set of tests, the scheme of having two Rbs with monobrachial homology was used. Again, chromosome 1 (the common arm) was the one under observation. As had been the case with sex-chromosome aneuploidies among offspring of irradiated males (see pp. 388-389), virtually all of the exceptional animals recovered by Cattanach et al. (5) were the result of chromosome loss in the irradiated parent, and loss can be the result of clastogenic, as well as malsegregation, events. Only one offspring was found who received both of its chromosome 1's from the treated mother, complementing a chromosome-1 loss in the nontreated tester-stock father. As the authors point out, this single case cannot prove X-ray induction of nondisjunction; it does show that autosomal nondisjunction--spontaneous or induced--can occur.

In past years, indications of spontaneous autosomal nondisjunction have been obtained for chromosomes 7 and 9 in (presumably) chromosomally normal animals. Thus, we observed 6 independent occurrences of d se/d se offspring from ++/++ x d se/d se crosses (in which well over 2 million F_1 have been recorded), and interpreted these as complementary nondisjunction types (37). Similarly a $c^{+}\ Hbb^{d}/c^{+}\ Hbb^{d}$ offspring was observed in a cross of $c^{+}\ Hbb^{d}/c^{+}\ Hbb^{d}$ x $c^{ch}\ Hbb^{s}/c^{ch}\ Hbb^{s}$ (38).

In comparing the two methods they used, Cattanach et al. (5) point out that, although the use of two Rbs with monobrachial homology is more effective than the use of a single Rb, the former scheme commonly suffers from male sterility, so that the induction of nondisjunction can be tested only in the male. In comparing the two-Rb scheme with the numerical sex-chromosome anomaly (NSA) method (data of Russell, see pp. 388-389), Cattanach et al. (5) found the latter to be more than twice as effective as the former in detecting aneuploid offspring of irradiated males. In comparisons of the single-Rb scheme with the NSA method, the latter was found to be about as effective as the former in detecting marked offspring resulting from irradiated zygotes, and about half as effective for detecting marked offspring of irradiated oocytes. It should be noted that all of these comparisons are for loss-type events, the bulk of which are probably the result not of nondisjunction, but of clastogenicity. The meaningful comparison--that for gain-type events--cannot yet be made from available data. Radiation is clearly a better clastogen than nondisjunction-inducer.

Tester stock in which nondisjunction is enhanced by interchromosomal effects. Another type of chromosomal constitution that has been shown to lead to elevated nondisjunction frequencies may be proposed for possible use in a tester stock: the presence of heteromorphic bivalents. A combination of 2 reciprocal translocations that involve the same two autosomes (Nos. 1 and 13), but have slightly different breakpoint locations, yields 2 sets of such heteromorphic bivalents (56). Females with this configuration exhibit a relatively high nondisjunction frequency for the overall chromosome complement. The authors suggest that disturbances in homology within the translocation bivalents cause many pairing and disjunction problems not only for the bivalents affected, but also for the normal chromosomes in the set.

In the case studied, the nondisjunction frequency was about 5 and 2%, respectively, for the short and long heteromorphic bivalent, and about 5% for normal bivalents. While nondisjunction of translocation chromosomes would not provide complementing gametes for the products of nondisjunction induced in chromosomally normal animals, the interchromosomal effect caused by heteromorphic bivalents provides an opportunity for detecting nondisjunction in the rest of the genome. The level found in the T(1;13) compounds (5%) is probably too low for practical use, but a considerably higher level might be achievable by addition of another set of heteromorphic bivalents. Some possible candidates for such another set may be available (40). The method would entail introduction of markers into a large number of the nontranslocated chromosomes.

Evaluation of complementation methods. Complementation methods, as do genetic methods in general, provide ease of scoring. Unlike cytogenetic tests, they can reliably measure loss as well as gain events. Complementation methods have the drawback that only a few of the products of aneuploid gametes are detected, namely those derived from fertilization events involving a complementary gamete supplied by the tester-stock parent. Furthermore, there is now evidence that when disomy for certain chromosomes comes in from the maternal side, the conceptus formed by fertilization with a corresponding nullisomic sperm may be inviable (Searle and Beechey, this

Volume). This would limit tests to those chromosomes that are not involved in this type of inviability phenomenon.

Robertsonian tester stocks provide the advantage of high nondisjunction frequencies for specific chromosomes. These, however, also lead to the production of many noncomplemented aneuploid gametes and thus to very low littersizes (41). Certain Rb combinations are male-sterile, a property influenced to some degree by genetic background. Tester stocks with heteromorphic bivalents would provide the potential of increased nondisjunction frequency for the entire genome. Many chromosomes would have to be marked, since for any one chromosome the increase would be small. Some possible combinations are, moreover, male-sterile or male-lethal. In such cases, nondisjunction induction could be tested in males only.

Direct Detection of Aneuploids

Genetic tests for sex-chromosome nondisjunction. The use of postnatally discernible genetic markers restricts direct detection of aneuploidies to those involving the sex chromosomes. Marker schemes have been devised (Fig. 2) that will detect supernumerary sex chromosomes as well as sex-chromosome loss, the general procedure having been named the numerical sex-chromosome anomaly (NSA) test (33-35). The simple scheme shown in Fig. 2 is suitable for detecting nondisjunction induced in males; a slightly more complex scheme is required for females. Although, in contrast to

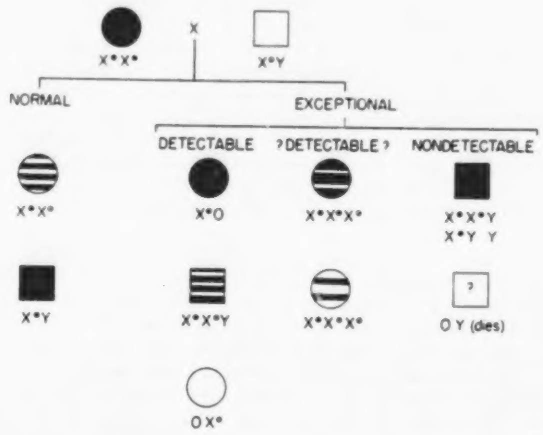

Fig. 2. Simplest genetic marker scheme for the detection of numerical sex-chromosome anomalies (NSAs). The female parent is homozygous for an X-linked gene (black circle) and the male carries a different allele at this locus (white square). Normal, heterozygous daughters are mosaic because of random-X inactivation (black-and-white-striped circle), and normal sons inherit the mother's allele (black square). External phenotype will unequivocally identify offspring resulting from loss of a paternal sex chromosome (X^MO, black circle), loss of a maternal X (OX^P, white circle) or inclusion of both paternal sex chromosomes (X^MX^PY, striped square); other types of nondisjunction progeny can be recognized with less certainty. This simplest marker scheme is suitable for detection of nondisjunction in males. A slightly more complex scheme must be used to detect nondisjunction in females. (Reproduced, by permission, from Ref. 35.)

cytogenetic procedures, the NSA method reliably measures loss (as well as gain) events, only supernumerary sex chromosomes can be considered as definitive evidence for malsegregation, since the XO type may be brought about also through clastogenicity or other mechanisms. The marker scheme can detect all survivors from trisomy-inducing events that occur in the primary spermatocyte or oocyte stage; its efficiency for detecting these events occurring in gonia or in the second meiotic division is ~67% or <50%, respectively. Scoring is not labor-intensive and does not require technical skills since exceptional animals are recognizable by their external appearance.

The NSA method has been used to detect nondisjunctional effects of radiation in various male germ-cell stages (34). In females, only predictyate stages (leptotene through diplotene) were explored with the full marker scheme (35). Markers used in irradiation of the dictyate allowed detection of loss but not of trisomy; the sensitive diakinesis stage of the primary oocyte has not yet been tested by the NSA method. Only 2 chemicals (triethylenemelamine [TEM] and methyl methanesulfonate [MMS]) have been investigated (both in males) by the NSA marker arrangement that allows detection of trisomies (34), and one of these, MMS, was reported to have produced XXY offspring (25). In tests of other chemicals, the crosses used were capable of detecting only chromosome loss (and not gain) events. The statement in a recent review (16) that "extensive genetic [NSA] tests failed to detect any significant increase in nondisjunction" is thus misleading.

Until the NSA method is more extensively compared with other methods for the detection of aneuploidy, it is not possible to determine whether or not sex-chromosome behavior is representative of that of other chromosomes in the complement. Several known differences between X-Y association and that of homologous autosomes could affect induced nondisjunction. The potential advantages of the method, aside from its nonlabor-intensiveness, are: (a) the very low spontaneous rates of sex-chromosome nondisjunction (<0.04%); (b) the fact that maternal and paternal events can be distinguished from each other by simple visual inspection; and (c) the fact that only germline nondisjunction is scored by certain ones of the trisomics, mitotic nondisjunction in the embryo being ruled out by the combination of genetic markers.

Use of markers to recognize trisomics in fetal stages: a proposed method. Before the systematic use of Robertsonian translocations (12,13) had revealed that probably no autosomal trisomy in the mouse survives more than very briefly after birth, we proposed marker schemes for the genetic detection of autosomal trisomics (32). One of these schemes is probably usable in fetal material, pending some preliminary exploration.

The treated parent would carry 2 closely-linked recessive markers in repulsion and would be mated to a tester stock that is homozygous for both markers, i.e., the cross is of the type *a*+/+*b* x *ab*/*ab*, which normally yields only offspring of phenotypes *a* or *b*, except for rare *a b* or + + crossovers. First-division nondisjunction (without crossover between centromere and markers; or, second-division nondisjunction after crossover) would yield trisomics of type *a*+/+*b*/*ab*, which are presumably + + in phenotype (Fig. 3). Chromosome counts for the exceptional wild-type fetuses would distinguish between nondisjunction and crossover events. Alternatively, the nondisjunction frequency could be estimated by simply subtracting the control frequency of the wild-type offspring. More than one chromosome might be marked in this manner, provided the phenotypes are not epistatic.

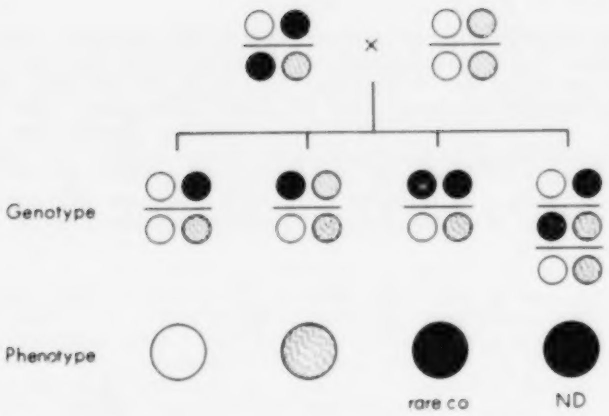

Fig. 3. Scheme for direct genetic detection of trisomics. The marker scheme involves 2 closely linked loci, represented by circles. The treated (or control) parent, carrying recessives (nulls) in repulsion, is mated to a doubly recessive, untreated animal. (Recessive at locus 1, open circle; recessive at locus 2, stippled circle; wild-type alleles at loci 1 and 2, solid circles.) Normal offspring are characterized by the recessive marker at locus 1 or locus 2. Trisomic offspring receiving 2 chromosomes from the treated (or control) parent will appear wild-type. Half the offspring derived from rare recombination between the two closely linked loci will also appear wild-type, but can be distinguished from the trisomics by chromosome counts. (The reciprocal crossover type is not shown.) Markers recognizable in fetuses have to be used in this marker scheme (see text).

Since trisomies for about half of the mouse autosomes survive past day 12 of gestation, it may be possible to use markers that affect eyes or eye pigment, and possibly markers affecting fore and hindlimb and/or tail development. A systematic search through lists of mapped mouse loci reveals a few possible candidate pairs. At least one such pair of closely-linked markers is located on an autosome whose trisomy is compatible with survival into late fetal stages.

This method has several features to recommend it. All of the products of gametes disomic for the marked chromosome are potentially recognizable. By contrast, with the use of Robertsonian tester stocks, only that fraction is recognizable that meets with a complementary nullisomic gamete from the tester-stock parent. If, for example, the Robertsonian had a nondisjunction frequency of 20%, as in the case of the "Rb" method described by Cattanach et al. (5), then only about 10% of the disomic gametes produced by the parent under study would be detected. The trisomy-marker method is thus an order of magnitude more efficient than this particular Rb scheme.

In addition, there is no restriction as to sex of the treated parent. By contrast, some of the complementing tester stock schemes (see pp. 384-387) can be used only for treated males, since the tester stock may be male-sterile or even male-lethal. In the trisomy-marker method, the diagnostic type is wild-type; no selection due to homozygosity of the recessive markers should operate against it.

The only drawback of the method is that it requires analysis of uterine contents rather than of living postnatal animals. On the other hand, this provides the opportunity of combining detection of nondisjunction with a routine dominant-lethal experiment and/or the postmidterm-death prescreen for trisomics (see pp. 381 and 383).

MAXIMIZING NONDISJUNCTION INDUCTION

In testing whether an agent has the capability of inducing nondisjunction, one would obviously wish to apply this agent under conditions that are optimal for its action. The effects of the following parameters on nondisjunction induction will be examined: germ-cell stage, age, and strain.

Germ-cell Stage

While the spindle apparatus and kinetochores are presumably potential targets for nondisjunction inducers at several stages of germ-cell development, events such as genetic recombination and meiotic pairing are vulnerable during a more restricted period. In the male, it is noteworthy that where positive effects of chemicals have been reported--e.g., with cyclophosphamide (27), ethyl methanesulfonate (EMS), MMS (48), or Ceresan (48)--the sensitive period includes preleptotene.

In the female, interference with genetic recombination (Grell, this Volume) or meiotic pairing (Moses et al., this Volume) should be achievable during the fetal period, probably days 12-15 pc; pachytene in most strains is already over by the time a female mouse is a day old and is entered into about day 15 pc (4). Surprisingly, however, it is the "preovulatory" stage that has repeatedly been found to be especially sensitive (16). This stage represents diakinesis/metaphase-1, and cannot involve any interference with chromosome pairing or meiotic exchange. It is, however, a stage particularly vulnerable to clastogenicity, as first shown almost 30 years ago (36). Irradiation of preovulatory oocytes has more recently been found effective in inducing chromatid interchanges in addition to other anomalies (31). Tease (50) has pointed out that this could lead to a scenario in which hyperploid pronuclei diagnosed at the first cleavage division would contain chromosomes that have been involved in an exchange. The linear increase in hyperploidy found by Tease might thus have been the result of clastogenicity rather than due to a radiation effect on segregation per se. It is interesting that no trisomy-inducing effect of radiation was found when dictyate oocytes were irradiated in the same study. (Possible confusions from clastogenic effects are also discussed on p. 380).

It would appear worthwhile to carry out a careful chromosome-banding analysis of presumed trisomic zygotes or embryos produced by exposure in the "sensitive preovulatory" stage. As long as doubt remains about whether hyperploidy induced by treatment at that stage is, in fact, the result of malsegregation, it seems advisable to include in experiments on females a group in which preleptotene oocytes (i.e., 12- to 13-day-old embryos) are treated. For treatment of males, the preleptotene stage (35 days between exposure and conception, or 12-13 days between exposure and cytogenetic scoring of secondary spermatocytes [26] should be included and has been figured in reported cases of chemically induced nondisjunction in mouse (3).

Age

With regard to spontaneous nondisjunction in mice, what little work has been done in males gives no evidence for an age effect (30). The considerable body of information on females has yielded conflicting results and is discussed elsewhere (Chandley, this Volume).

The effect of age can be very strong in females heterozygous for Robertsonian translocations; thus, for Rb(1.3)1Bnr, the rate of Rb nondisjunction changed from 49% at 7-10 weeks to 73% at 11-13 months (14). Working with two other Rbs, Lyon et al. (20) found the highest nondisjunction frequencies to occur in middle age (5-7 months) in one and in old age in the other. Similar differences have been reported between chromosomally normal strains (see Ref. 3 for review), but the numbers on which the conclusions are based are often very small. One interesting finding made both by Martin et al. (21) in M2 analysis and by Fraser and Maudlin (8) in first-cleavage studies of chromosomally normal mice is that the age-related increase occurs only in trisomy and not in monosomy. This suggests either an age effect on preferential segregation of the disomic to the egg (vs polar body), or the coming into play of some factor other than nondisjunction (e.g., extra replication of chromosomes).

With the objective of finding conditions that optimize tests, it is of interest to examine whether the induction of chromosome malsegregation is affected by increasing age. The only agent tested in this regard is ionizing radiation, and, again, the evidence is conflicting. Some investigators have suggested that oocytes of old females are more sensitive than those of young ones (53,59). Others have been unable to show an increased sensitivity of old females, or, in fact, the ability of radiation to induce nondisjunction (23,31,44,45). A recent careful study of first-cleavage metaphases (50) found a linearly dose-related radiation effect on frequency of hyperhaploid pronuclei in both young and old females when irradiation was administered in diakinesis of the primary oocyte (possibly clastogenic effects). Although there was a significant age effect on spontaneous hyperploid frequency, the radiation slopes were the same in young and old females, i.e., there appeared to be no increase in radiation sensitivity with increasing maternal age. There is thus, at present, no evidence to indicate that nondisjunction induction may be optimized by treatment at any particular age.

Strain

There is to date no clear indication of strain differences in frequency of spontaneous nondisjunction of normal chromosomes in males (30) or females (3,17,22) when observations are made at M2 or in the zygote. This frequency is in the 1-2% range in most studies. The frequency of sex-chromosome univalency in primary spermatocytes has been reported to be under genetic control (2), but the relation between univalency and eventual aneuploidy is still unclear (see pp. 379-380). It has been suggested that strains may differ with regard to the presence or magnitude of a female-age effect on nondisjunction (18,21; Chandley, this Volume); however, more extensive evidence is needed on this point.

On the other hand, there appear to be clear differences between Robertsonian translocations with regard to nondisjunction frequency (20), a parameter that could enter into the design of genetic complementation experiments where such translocations are used in tester stocks (see pp. 384-387). The evidence is also strong for strain differences in the length of survival of trisomics (12), the maternal genotype probably being the governing one (52). Experiments involving observations during the late embryonic or fetal periods may need to take this variability into account. No strain comparisons have been made for frequency of induced nondisjunction.

ACKNOWLEDGEMENTS

Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-840R21400 with the Martin Marietta Energy Systems, Inc.

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DISCUSSION

HANSMANN: There are no clear-cut data in the literature comparing effects in different species. But we have evidence from recent experiments that the mouse may underestimate the effects of chemicals. The mouse has very synchronous segregation during meiosis, which may produce predominantly diploidy. A species having more asynchronous segregation during meiosis may be more suitable for analyzing induced nondisjunction.

RUSSELL: Unfortunately, the most common system for measuring nondisjunction in various species has been metaphase-2 analysis, which I feel can be unreliable, and may measure what is basically clastogenic damage rather than (or along with) malsegregation.

HANSMANN: I agree very much that spermatocytes are very difficult because of the fuzziness of chromosomes. But if you have enough experience, you will get perfect oocyte chromosomes.

RUSSELL: One thing that has puzzled me is that the "sensitive preovulatory stage" in the female has been used so much for treatment. That is a stage at which you no longer can interfere with pairing or recombination--events that are potentially vulnerable for inducing nondisjunction. Treatment just before ovulation, i.e., already in diakinesis, could yield effects predominantly related to clastogenicity.

HOOK: Would you comment on radiation induction of nondisjunction in experimental animals? Can the inconsistencies be explained?

RUSSELL: The experiments that seem to be conflicting have varied very much according to stage of treatment. If recombination or synaptic events are important in meiotic nondisjunction, one would have to irradiate either at that time, or preceding that time. The female mouse would have to be treated 12-14 days p.c. Very little work, indeed, has been done with those stages. In the male it is noteworthy that wherever induction of trisomies has been demonstrated, the treated stages have always included what is probably preleptotene. There has been more conflict, I think, about possible age effects on radiation-induced nondisjunction than about anything else. Again the stages of treatment have varied. In what I consider to be a very reliable experiment, Tease recently showed clearly dose-related radiation effects on hyperploidy scored at the first cleavage, but no age effect on radiation induction.

RESEARCH NEEDS IN MEIOSIS, MECHANISMS OF SYNAPSIS, AND CHIASMA REGULATION

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INTRODUCTION

The conspicuous features of meiosis are the pairing of homologous chromosomes, the high levels of chromosomal recombination, and the reductional segregation of homologous centromeres. Through these processes of synapsis, recombination, and segregation, meiosis results in the formation of genetically diverse haploid cells. Of these processes the mechanism of recombination is well understood through the Holliday model and later modifications (5). The interaction between recombinant events which results in genetic or chiasma interference is not evident from the molecular models and still needs to be explained (2). There are a number of models for homologous chromosome synapsis and disjunction but the experimental verification has been difficult. New insights have come from observations on the behavior of synaptonemal complexes (SCs) in unusual pairing situations and of rearranged chromosomes by Moses and Poorman (4) and von Wettstein et al. (7). It appears that the processes of synapsis, interference, and disjunction are under complex structural and species-specific regulatory controls which make it unlikely that generalization can explain every case. For example, in general, homologous synapsis is a prerequisite for orderly disjunction, yet Hymenopteran males manage meiosis with a single set of chromosomes. Similarly, achiasmic Dipteran males accomplish orderly disjunction in the absence of SCs and recombination. Meiosis usually produces haploid cells, but in parthenogenic yeast it produces 2 diploid cells, and in some parthenogenic insects and amphibians meiosis produces triploid cells.

Interference with normal meiosis, which results, among other things, in aneuploid meiotic products, is the topic of this Volume. I will combine this topic with my interest in the mechanisms of chromosome synapsis, and will describe the unusual and revealing conditions that cause unbalanced segregation in meiosis of the grasshopper *Chloealtis conspersa*.

ANOMALOUS ASSOCIATIONS

Figure 1a shows an early anaphase 1 where the normal segregation of bivalents 7 and 8 is disturbed by the presence of an anomalous chiasma between chromosomes 7 and 8. In Fig. 1b the anomalous chiasma between chromosomes 7 and 8 interfered with the normal chiasma between the two

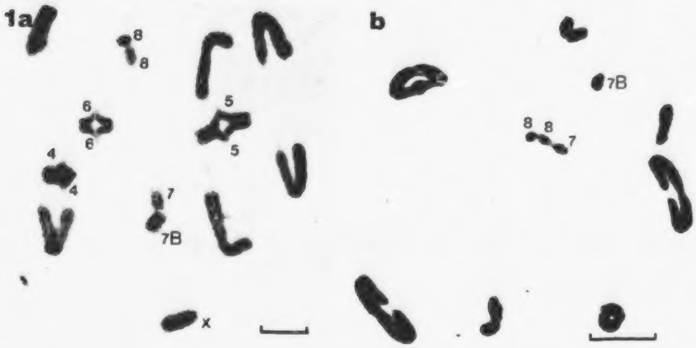

Fig. 1. Aneuploidy in grasshopper meiosis. (a) The anomalous association between the distal portions of chromosomes 7 and 8 results in a misalignment of the chain of 4 chromosomes at early anaphase 1. One cell receives two chromosome 8s and the other two chromosome 7s. In most spermatocytes with 7=8 associations disjunction is normal. (Cell from 53CC6I, bar = 5 μm.) (b) The anomalous association between chromosomes 7 and 8 apparently interfered with the customary chiasma between the two chromosome 7s. As a result, chromosome 7 with the supernumerary B segment is unpaired. Sometimes chromosome 8 occurs as a univalent. Both are rare events. The short arms of the metacentric chromosomes of the bivalent at top left have an infrequent interstitial chiasma. (Cell from 55DD2-146A, bar = 5μm.)

chromosome 7s, so that a no. 7 univalent results. Teshima (6) found that in Chloealtis all chromosomes, except the no. 5 pair, are capable of forming such distal anomalous associations with each other; her summary, modified for this presentation, is shown in Fig. 2j, with examples in Fig. 2a-i.

The elements capable of forming anomalous synapsis and a chiasma are labeled a and d in Fig. 2j, and supernumerary chromosome segments (B) are shaded. A given individual may have any combination of homologs, for instance, 7/7, 7/7a, 7a/7a, or 7a/7B, etc. Figure 2a and b demonstrate an anomalous association of type 7B/7a=8ad/8ad (the equal sign marks the association); Fig. 2c and d show an association between 2 metacentric bivalents, M/Md=Md/M (M stands for metacentric chromosome); and Fig. 2e and f show a complex arrangement, 7B/7a=8ad/8ad=Md/M. The anomalous associations are also detectable at the electron microscope level where they appear as anomalous SCs between the ends of heterologous chromosomes.

Figure 3 gives 2 examples of anomalous SCs between the distal portions of chromosomes 7 and 8. Figure 3c shows an entire set of SCs as well as an anomalous SC drawn to scale horizontally but not vertically. Similar SCs between heterologous chromosomes have been reported by Bojko (1) in human oocytes, and this suggests that these phenomena have significance beyond Chloealtis.

In a sample of 35 males collected in the Toronto area, Teshima (6) found that 0.138% of the cells had anomalous associations (54,224 metaphase 1 cells). The number was significantly higher in 20 8/8B males (0.537%; 38,186 cells). I found an unusually high frequency of 7=8 associations among the progeny of a sib mating of a 1983 Chatham, Ontario collection. Of the 21 sons, 9 had 7=8 associations at levels of 8% to 86% of their metaphase 1 cells. For example, male 53I had 80% 7=8 associations as well as other associations shown in Fig. 2a, c, and e. The electron micrograph of an anomalous SC of male 53F is shown in Fig. 3a. Analysis of the data suggests that the original sib mating was of type 7B/7B 8ad/8- x 7a/7- 8ad/8-. A number of F1 sib matings together produced 16 males, each with 2 normal chromosome 7s, 16 males, each with 2 chromosome 7Bs, and 34 males heteromorphic for 7-7B. Anomalous associations between chromosome 7 and 8 were found in 21 of the 34 males heteromorphic for 7-7B. No associations were found in the 32 males homomorphic for chromosomes 7 and 7B.

There are 2 conclusions from the above observations. First, chromosome 7-7B heteromorphism is necessary for associations between chromosomes 7 and 8 to be expressed in this family. The explanation may be simple: the 7B segment disrupts distal pairing between the no. 7 homologs and thereby releases the 7a segment, which can then pair with 8a (6). Second, since not all males heteromorphic for chromosome 7 form associations, the condition is necessary but insufficient. The additional requirement resulted in the formulation of hypothetical synaptic units *a* and *d*. Thus, associations between chromosomes 7 and 8 occur in males 7B/7a 8ad/8-, but not in males 7B/7a 8-/8-, 7B/7- 8ad/8-, or 7a/7a 8ad/8-.

SYNAPTIC INITIATION

The synaptic unit is a hypothetical hereditary unit, operationally defined as a segment of a chromosome capable of initiating synapsis and chiasma formation. In *Chloealtis* the units are recognizable by the anomalous associations between heterologous chromosomes. The units are hereditary entities judging by the presence of anomalous associations in some families, but not in others. The pattern of inheritance within a family shows that the expression of at least one of the units is hypostatic to chromosomal heteromorphism. The synaptic unit is probably only a small chromosome segment. This is suggested by the extreme distal chiasma position of the anomalous association (Fig. 1 and 2). It is also evident from the anomalous SC which always occupies the terminal 1 µm of the chromosomes (Fig. 3b, 1 µm), but may extend a variable length in a proximal direction (Fig. 3a, 3.4 µm). In 19 pachytene cells of one F2 male, E21DDδE, the lengths of the 7=8 anomalous SCs were 0, 0, 0, 0, 1, 2, 2, 2, 4, 4, 5, 5, 5, 5, 6, 7, 8, 10, and 10 µm. Evidently only the most distal portion is significant in chiasma formation, and the remainder of the anomalous SC contains heterologously synapsed portions of chromosomes 7 and 8, which are incapable of chiasma formation.

A small size is further suggested by the essentially normal SCs between chromosomes heterozygous for synaptic units *a* and *d*. There is no sign of a mismatch in the distal SC segment of such a bivalent. More informative is the occasional anomalous association distal to the customary distal chiasma of a metacentric bivalent (Fig. 2e and h). The usual distal chiasma localization in metacentric chromosomes is shown by the 3 ring bivalents in the oocyte metaphase 1 of Fig. 4, and a male metaphase 1 in Fig. 5a.

When a metacentric chromosome is involved in an anomalous association, the association interferes with the normal chiasma in that arm and a chain of chromosomes results (Fig. 2c and e). Occasionally, however, the distal chiasma is intact and even more distal is the anomalous association (Fig. 2g and h). Fig. 2i shows that the *d* synaptic site of chromosome 8 and the metacentric must be extremely small not to interfere with the normal distal chiasma between the metacentric arms. The same small size and close juxtaposition of two different synaptic sites are evident from the proximity of

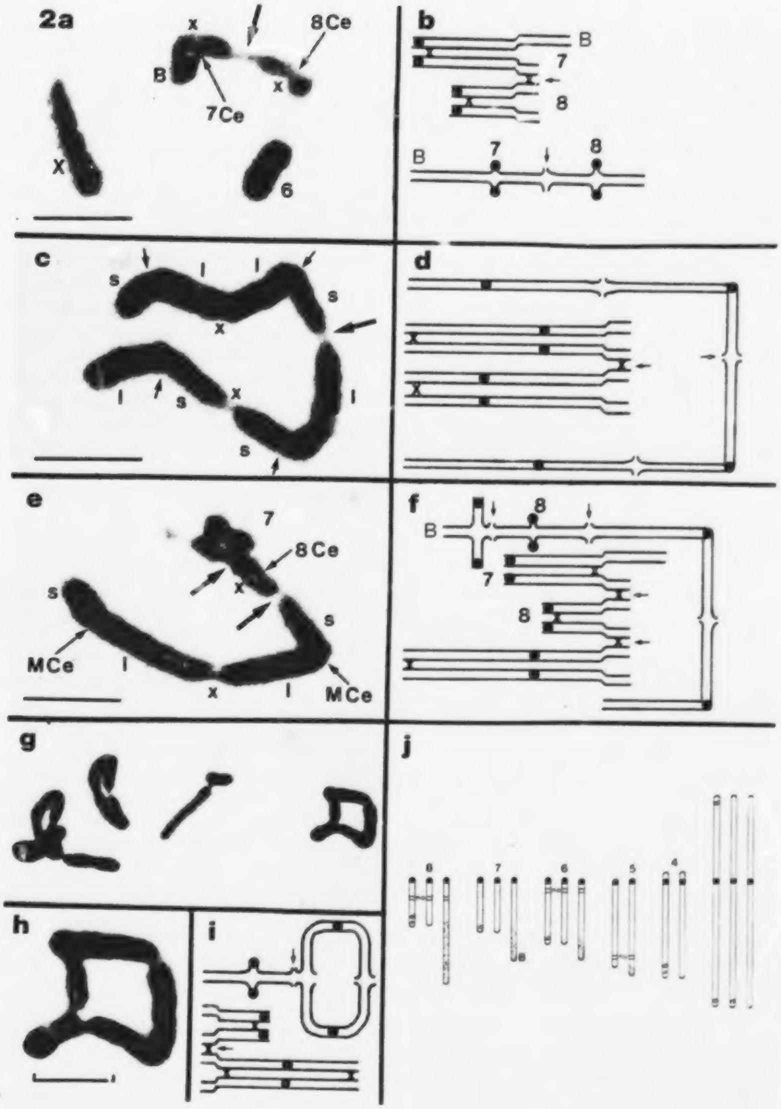
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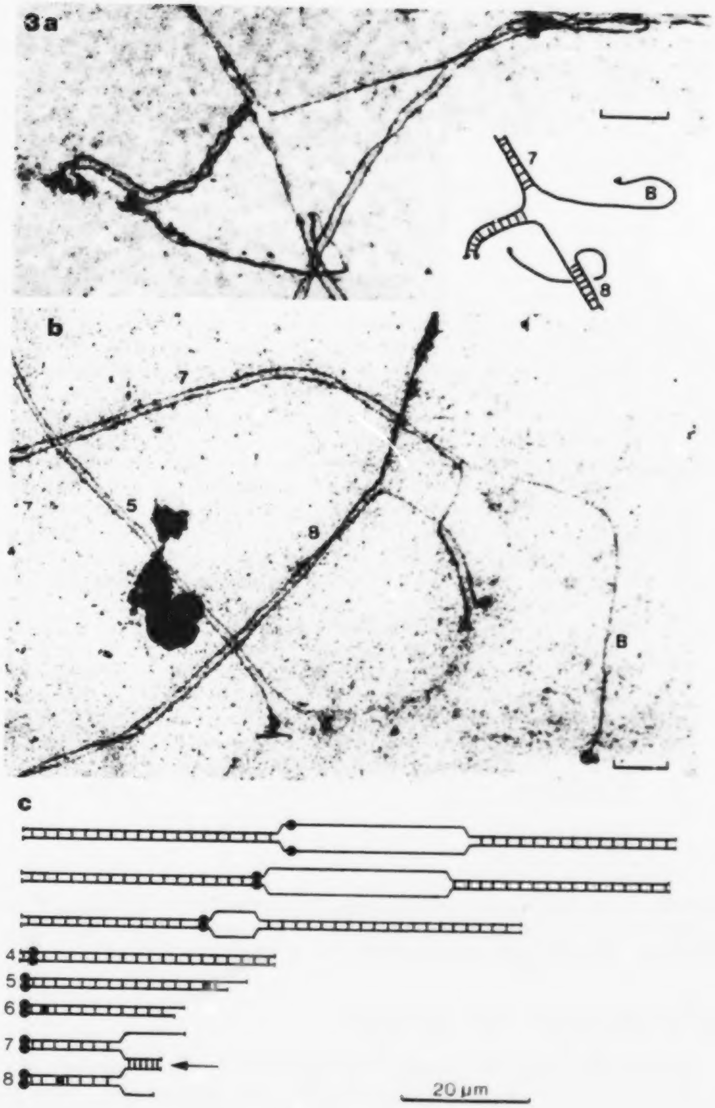
Fig. 2. Anomalous associations in *Chloealtis*. Light micrograph legend: X = X chromosomes; B = supernumerary chromosome segment; Ce = centromere; small arrows mark the centromere position; fat arrows point to the anomalous association; x = position of a chiasma; l = long arm; s= short arm. Bivalents are identified as 6, 7, or 8, or M for metacentric. All scale bars represent 10 μm. Fig. 2a, c, and e are from E 536I. Fig. 2g and h are from E53CC 3-246K. Diagram legend: B = supernumerary segment (shaded in 2j); arrows point to the anomalous associations; chromosomes and bivalents are numbered 8, 7, 6, etc.; no = nucleolus position; closed circles represent centromeres; x = chiasma position; *a* and *d* are synaptic units. (a,b) An anomalous association between the distal segments of chromosomes 7 and 8. The upper diagram illustrates the chromatids at pachytene with the positions of the proximal chiasmata between synapsed chromosome 7s and between chromosome 8s, and the distal anomalous association with a chiasma between chromosomes 7 and 8. The derived metaphase 1 configuration is drawn below. (c,d) An anomalous association between 2 metacentric bivalents. The chromatid diagram of pachytene shows that the anomalous association has interfered with the customary distal chiasmata in the same arm. The metaphase 1 configuration is drawn around the pachytene diagram. (e,f) Anomalous associations between bivalents 7, 8, and a metacentric bivalent. These demonstrate that both chromosome 8s are capable of forming anomalous associations. The chromosome 7s have a distal chiasma, and more distal is the anomalous chiasma with the chromosome 8s. This is taken as evidence that the synaptic unit responsible for the anomalous association is of small size. The diagram illustrates the chiasma positions at pachytene and the chain of 6 chromosomes at metaphase 1. (g,h,i) The rare case of two closely juxtaposed chiasmata in the distal portion of a pair of metacentric chromosome arms and in an anomalous association distal to it. (j) A summary diagram of the chromosomal polymorphisms in *Chloealtis*. Only one set of metacentric chromosomes is shown.

chiasmata in chromosome 7 and between chromosomes 7 and 8 in Fig. 2e and f. The positions of these chiasmata also prove that the homologs are truly homologous immediately proximal of the *d* synaptic unit.

SYNAPTIC EXTENSION: "LAX SYNAPSIS"

The formation of a crossover requires the juxtaposition of homologous DNA sequences with molecular accuracy. In *Chloealtis* there is evidence that the extension of synapsis is less than accurate and occasionally nonhomologous. Such synapsis does not permit crossover events and thereby "regulates" crossing-over. The hypothesis is examined that "lax synapsis" regulates the frequency and distribution of chiasmata. The evidence for synapsis between heterologous chromosomes or chromosome segments has been reported widely and is referred to as "heterosynapsis" by Moses and Poorman (4). In *Chloealtis* heterosynapsis is exemplified by extension in a proximal direction of distal anomalous SCs (Fig. 3), and by the matched SC ends of heteromorphic chromosomes (Fig. 5). In the latter case heterosynapsis fades into homosynapsis (Fig. 5c and d).



The strongest evidence for lax synapsis is the observation that even homologous chromosomes are less than perfectly aligned along the SC (Fig. 6). The misalignment of homologous chromosomes along an SC is evident from the mismatched centromeres in Fig. 6. The two cells from the same male show a range of centromere (mis)matches from 0 to 3 µm apart. Occasionally synaptic initiation can be seen in the interstitial regions of metacentric chromosomes (not shown) and occasionally interstitial chiasmata occur as well (Fig. 1b). It follows that there are, in fact, interstitial synaptic initiation sites, but they are usually not expressed.

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Fig. 3. Anomalous synaptonemal complexes (SCs). (a) An anomalous 3.4 μm SC between one element from SC 7 and one element from SC 8. The remaining unpaired element of SC 7 contains the supernumerary B segment. (E53♂F cell 23, bar = 1 μm.) (b) A 1.4 μm anomalous SC between the distal parts of chromosomes 7 and 8. The unpaired element of the chromosome 7s contains the supernumerary B segment. The dense body is probably the distal nucleolus of chromosome 5 but the attachment is not well defined. One chromosome 5 has a small distal B segment in this male (E21DD♂E, bar = 1 μm.) (c) A diagram of a *Chloealtis* SC spread, drawn to scale in the horizontal dimension. Frequently the central portions of the metacentric chromosomes are not paired. In this cell chromosomes 5, 6, and 7 are heteromorphic. There is an anomalous association between chromosomes 7 and 8 (arrow). Solid circles represent centromeres, solid squares are nucleolar organizers.

CHIASMA INTERFERENCE

In *Chloealtis* bivalents 7 and 8 normally have one chiasma each. When 7 and 8 are anomalously associated there are 3 chiasmata (Fig. 2a and b). Rarely is there interference (Fig. 1b shows interference). A metacentric bivalent normally has 2 chiasmata. An anomalous association between bivalent 8 and a metacentric only has 3 chiasmata instead of 4. The anomalous association preempts the formation of a chiasma anywhere else in the same arm of the metacentric (Fig. 2c, d, and f; the exception is Fig. 2h and i). Thus, there is nearly complete chiasma interference in the metacentric arm. This behavior correlates well with the pattern of synaptic initiation. In *Chloealtis* and other grasshoppers, telocentric chromosomes are attached with both ends, centromeric and telomeric, to the nuclear envelope. The metacentrics are attached at the telomeres but not at the centromeres. Synapsis usually starts near the envelope.

A MODEL

The observations on synaptic initiation and lax synapsis can be combined to design a model that explains chiasma distribution and chiasma interference in *Chloealtis*. This model proposes the existence of synaptic units which are small chromosome segments capable of initiation, homologous

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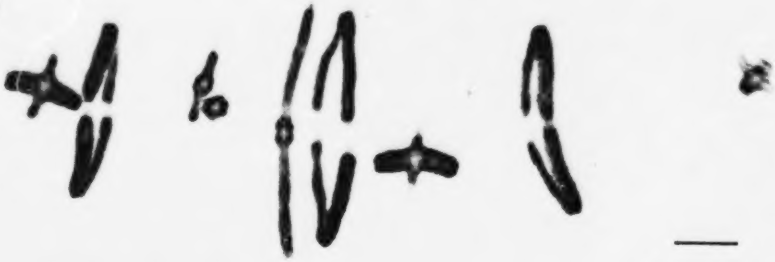

Fig. 4. Oocyte metaphase 1 bivalents show that the regulation of chiasmata number and position is similar in male (Fig. 5a) and female *Chloealtis*. In particular, the metacentric chromosomes usually have distal chiasma localization. (Bar = 10 μm.)

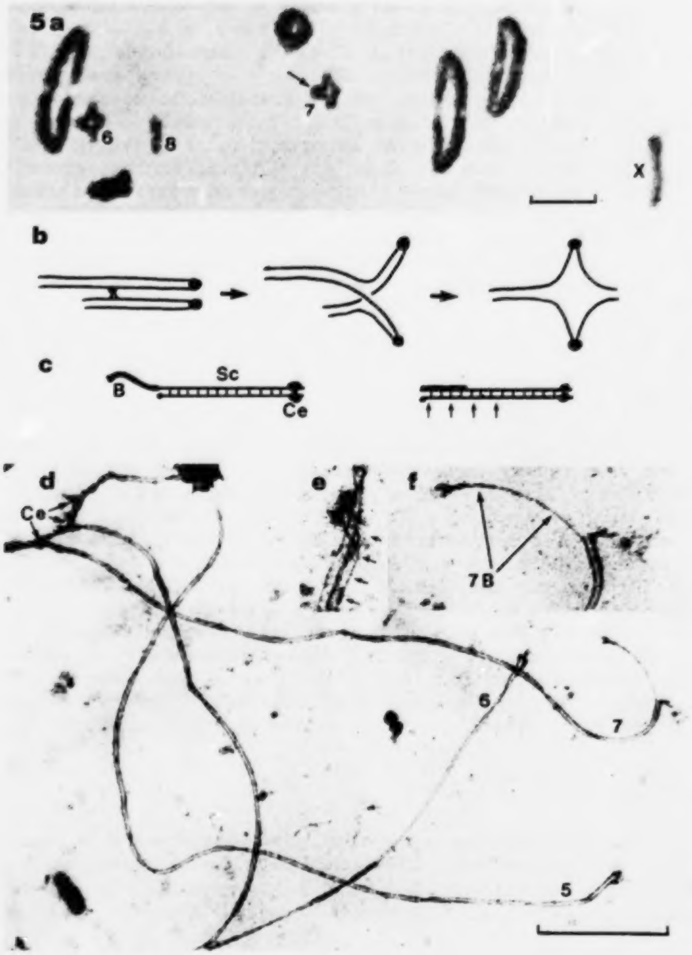

Fig. 5. Heterosynapsis. Legend: SC = synaptonemal complex; Ce = centromere; B = supernumerary segment. (a) Evidence for heteromorphic chromosomes 6 and 7. The bivalents are asymmetric and the arrow marks the supernumerary B segment of chromosome 7. (Bar = 5 μm; E276B.) (b) The chromatid diagram illustrates the origin of the asymmetric bivalent in 5a. (c) The SC diagram illustrates that with homosynapsis the B segment should not be part of the SC. With heterosynapsis the SC has matched ends but the distal segments are not homologous. (d,f) The SCs 5, 6, and 7 of cell 17 E21DD6M. The B segment is not a part of SC 7. (e) The matched ends of SC 7 of cell 14 E276F which is heteromorphic for chromosomes 7 and 7B.

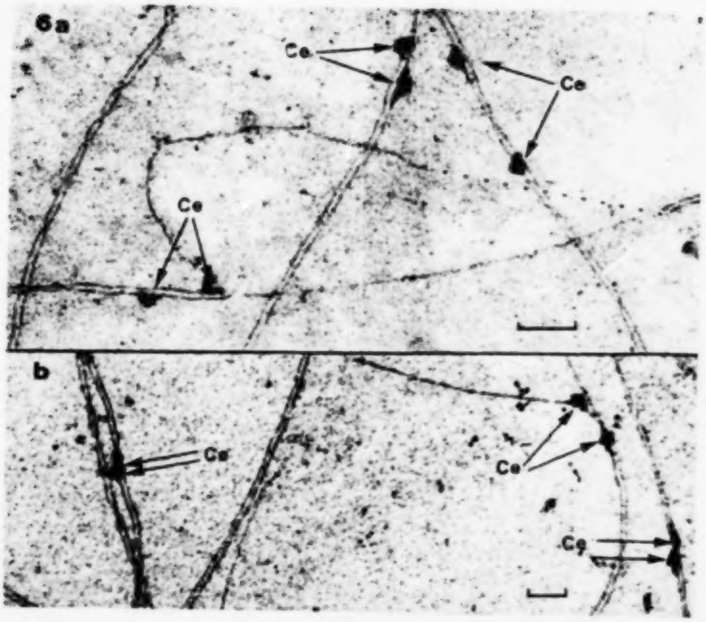

Fig. 6. Lax synapsis. Evidence that homologous synapsis is less than perfect. (a,b) The centromeres of the homologous metacentric chromosomes are expected to be juxtaposed if synapsis is strictly homologous. Varying degrees of mismatch are evident indicating that synaptic accuracy is rather lax in these regions. Since no chiasmata can form in mismatched regions, "lax synapsis" may in part regulate the distribution of chiasmata. (E21DD60 cells 01 and 04. Bar = µm.)

synapsis, and reciprocal recombination. It also postulates that SC formation extends from the initiation site(s) in an undiscriminating manner as far as homology is concerned. Potential initiation sites that have not established synapsis are rendered ineffective by lax synapsis.

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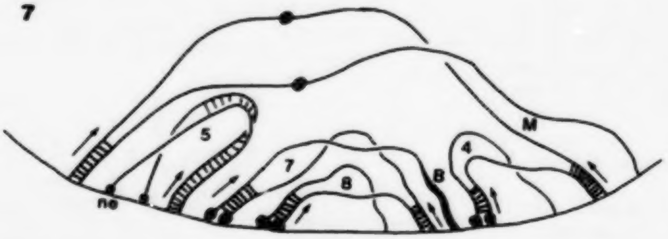

Fig. 7. Model of synapsis and chiasma regulation in Chloealtis based on the existence of synaptic initiation sites and lax synapsis. Details discussed in text.

This model meets the basic demand for at least one chiasma per bivalent. The numbers and positions of additional chiasmata depend on the species-specific distribution of synaptic initiation sites and on development of the SC. Since the model postulates that lax synapsis extends away from an effective synaptic initiation site, it follows that the chance of an additional chiasma near the initiation site is small. The model thus gives an explanation for chiasma interference based on the observed lax synapsis of homologous chromosomes.

Fig. 7 interprets the observed chiasma interference in terms of effective synaptic initiation followed by lax synapsis. In the diagram, bivalent 5 started synapsis at the distal end and lax synapsis extends towards the centromeric end, preventing further chiasmata along the length of the arm. The result is one distal chiasma, such as can be seen in Fig. 4. In many metaphase 1 cells, bivalents 4 and 5 have a proximal and a distal chiasma and synapsis apparently can initiate at both ends (Fig. 5a). Bivalents 6, 7, and 8 are the shortest and normally have one chiasma each (Fig. 4 and 5). Presumably initiation occurs at one end and lax synapsis reaches the other end before it can initiate synapsis. Fig. 7 further illustrates the case where distal synapsis of bivalent 7 is obstructed by the supernumerary 7B segment. Here the distal segments of chromosomes 7 and 8 can pair if they share a synaptic unit a. Apparently heterologous SC extension from a is difficult since no anomalous 7-8 SC was found longer than 1/2 the length of no. 8. Since lax synapsis from the anomalously paired distal end does not reach the centromeric end, synapsis can also be initiated at the free centromeric ends. The result is 3 chiasmata without interference. Fig. 7 further shows an association between a metacentric (M) and chromosome 4. The customary synaptic initiation site of the metacentric arm is inactivated by a lax synapsis from the d-d initiation site, leaving the metacentric arm free from additional chiasmata.

With the genetic identification of synaptic units and the recognition of lax synapsis it will be possible to analyze the synaptic units and the nature of SC extension in detail. It is expected that eventually it will be possible to verify these hypotheses through the construction of strains with predetermined patterns of synapsis and chiasma formation.

ACKNOWLEDGEMENTS

Dr. K. Rothfels (Department of Botany, University of Toronto) collected the Chatham, Ontario Chloealtis, and assisted with the design of laboratory rearing procedures and with the light microscope (LM) cytology. Barbara Spyropoulos helped with the rearing and LM cytology. Cecilia Moens did the EM preparation and analysis. Les Kohalmi did the cytology of female grasshoppers. This research is supported by the National Research Council of Canada.

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DISCUSSION

NICKLAS: What is the evidence that these are, in fact, nonhomologous? Might not there be some homology between the ends of 7 and 8?

MOENS: Oh yes. The chromosome 7 is a different chromosome than 8. So when I say nonhomologous chromosomes, the segments themselves are presumably homologous.

STERN: I would like to mention a very clear case in wheat, which of course you know has three sets of chromosomes. Each of these sets has a great deal of homology with the others, but they manage, under normal conditions, to keep apart. It was the Carlsberg group about three years ago that showed that when meiosis first begins, you get a lot of what we call homeologous pairing between different members of the sets. But those are corrected before the actual crossover begins.

GREEN: I would like to make a statement about the association of the ends, because there are some data from Drosophila that suggest there may be a very, very common feature to the ends which is not the telomere. At least two different people have isolated what they call telomere clones. These are cloned DNA which, when you in situ hybridize, goes to the ends of the chromosomes. Some years ago we found a mutant called μ2. It is a very interesting mutant because it allows you to lop off the end of the chromosomes, especially with X-rays. Other telomere clones do not in situ hybridize anymore. Yet, so far as we can tell, these chromosomes replicate very well, and therefore there must be some end.

MOSES: The interesting point is that comparing an inversion in the mouse with an inversion in the grasshopper that Mike Dresser has worked on, the one in the grasshopper is sloppy indeed. There is nonhomologous pairing that clearly occurs early. There is asynapsis that persists late and there is desynapsis that is hard to distinguish from asynapsis, etc.

MATERNAL AGING AS THE IMPORTANT ETIOLOGICAL FACTOR IN HUMAN ANEUPLOIDY

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INTRODUCTION

The graph shown in Fig. 1 illustrates the steep and dramatic rise in Down syndrome conceptions that occur in man as maternal age advances. No other single factor is as important in the etiology of this particular trisomic condition.

The association between maternal age and Down syndrome was described by Penrose in 1933 (22), long before the chromosomal basis of the syndrome was discovered. At maternal age 20 yr, the incidence of Down Syndrome births is about 1 in 2,300 (0.04%); at ages 30-34 yr, about 1 in 880 (0.1%); and at age 45 yr and over, about 1 in 30 (over 3%) (12,26).

In fact, trisomies for most autosomes in man are associated with increased maternal age, the magnitude of the effect varying, however, among individual chromosome pairs. Data from spontaneous abortions show that, in general, the effect is strongest for the smallest chromosomes, with the exception of trisomy 16 which, while being the most common trisomy among spontaneous abortions, is only weakly age-related (13). The continued accumulation of data from spontaneous abortions, together with that from livebirths, make it possible now to calculate age-specific rates for total trisomy in clinically recognized conceptions (12). Assuming a 15% spontaneous abortion rate among all recognized pregnancies, the data show that the estimated likelihood of trisomy among women 20 years of age is only 2%, while for women 42 years and older it rises to 35%. Indeed, as Hassold and Jacobs (12) point out, the majority of oocytes produced by women in the oldest age group will, on the basis of these figures, be expected to be aneuploid, since it is assumed that nondisjunction leading to trisomy will lead also to monosomy on at least the same scale. Monosomic embryos, it is thought, will be lost, however, very early in pregnancy and will not produce recognizable pregnancies.

In consideration of these facts, there is, therefore, a need to identify some etiological factor associated with the aging process in the female that is affecting a very high proportion of eggs ovulated during the last years of the reproductive span, and ever-increasing numbers of eggs during the reproductive years leading up to this time. It is to this end that research has been carried out in our laboratory over the past decade. In spite of the variety of proposals that have been put forward to

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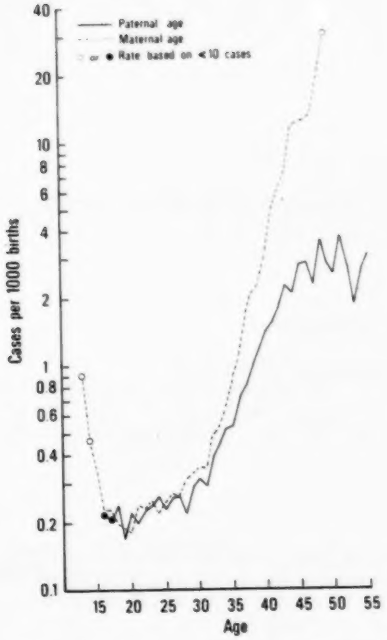

Fig. 1. Down syndrome rates by parental age (from J.D. Erickson [1978] Ann. Hum. Genet. 41:289-298).

account for the maternal age effect for trisomy in man (see Ref. 3 and 12 for review), no single factor remains as incontrovertible as aging itself, and it is to this process, therefore, that we have directed our attention.

Some recent experiments using CBA mice attempted, first of all, to separate the influence of chronological age per se from that of biological aging in the ovary (4). This particular strain of mouse was chosen because it has a short reproductive span compared with that of many other strains, and shows an age-dependent increase in aneuploidy (8,11). Furthermore, the CBA ovary is virtually depleted of oocytes at the end of reproductive life (9), as is the ovary of the human female (7).

Unilateral ovariectomy (uni-ovx) was performed on a group of CBA mice at the beginning of their reproductive lifespan. Removal of one ovary from young adult mice results in compensatory ovulation from the contralateral ovary. The long-term consequences of uni-ovx include fewer subsequent estrous cycles and an earlier onset of acyclicity, though the pattern of change--first to irregular cycles and then to acyclicity--is similar to that seen in intact animals. In earlier work (2), it was shown that the rate of utilization of oocytes from the store of primordial follicles is the same in uni-ovx females as in intact controls. It seems, therefore, that it is the overall size of the follicle population that limits ovarian lifespan, and that acyclicity probably results when a critical threshold number of large secretory follicles, required for maintaining the positive feedback mechanism for pituitary gonadotrophin release, is obtained.

The aim of our study was to advance the process of biological aging by unilateral ovariectomy, and to determine whether this brought about a correspondingly early onset of age-related aneuploidy. In fact, an earlier increased incidence of aneuploid 3.5-day embryos in aging mice was observed after uni-ovx, and as far as we are aware, this was the first demonstration that the occurrence of age-related aneuploidy can be experimentally modified by manipulating the normal pattern of physiological aging of the reproductive system. A significant potentiating effect of uni-ovx on aneuploidy was found in animals aged 5-6 mo, and in all females aged up to 9 mo; while only one trisomic embryo was found in controls, 5 were recorded in the uni-ovx group (Tab. 1).

The results of this experiment suggest that the abnormal segregation of meiotic chromosomes leading to aneuploidy is an epiphenomenon of the process of physiological aging of the reproductive system which is manifested in the changes of the estrous cycle patterns. The findings cast doubt on the postulated significance of factors that are dependent on chronological age per se.

The results could not provide a specific hypothesis of what aspect of ovarian aging was primarily at fault, but the implications for human aneuploidy, and Down syndrome in particular, are worth considering.

First, they would imply that unilateral ovariectomy performed early in life may be an additional risk factor for Down syndrome. Also, it could be speculated that the probability of conceiving a trisomic fetus will be determined primarily by the size of the remaining oocyte store and, hence, by the time of the approaching menopause.

One interesting group of women displaying early biological aging and conforming well to our model are those rare individuals with Turner syndrome (XO) who manage to achieve pregnancy during their brief reproductive span. Menopause in such women occurs generally in the late 20s, but for their chronological age, they show a remarkably high level of fetal wastage and a greatly increased risk of producing Down syndrome offspring (17,25).

A logical extension of our hypothesis is that any factor, idiopathic or environmental, that depletes the oocyte population, could advance the maternal age effect for aneuploidy. Smoking, for example, is known to advance the age of menopause (15,16) and an increased incidence of trisomic abortion has been observed in older women who smoke (18).

The above studies point to some factor operating in the adult female as the underlying cause of age-related aneuploidy. The alternative "production line" hypothesis (14) still remains, however, to be disproved.

Tab. 1. Chromosome anomalies of 3.5-day embryos in CBA/Ca mice: variation according to maternal age and unilateral ovariectomy.

Experimental group	Maternal age (days)	Total embryos analyzed	% analyzed	Chromosome analysis								% aneuploidy
				4n	3n	2n/3n	41	40	39	38	37	
OVX	63-91	67	29.0	-	-	-	-	59	8	-	-	11.9
SHAM	63-91	66	27.2	-	1	-	-	61	4	-	-	6.1
OVX	154-182	75	29.2	-	1	-	3	60	10*^{1}	1	-	17.3
SHAM	154-182	65	37.1	-	-	-	-	62	3*^{2}	-	-	4.6
OVX	245-280	41	28.1	2	-	1	2	25	7*^{2}	3	1	22.0
SHAM	245-280	58	24.9	-	1	-	1	45	6	3	2	12.1
SHAM	308-350	46	33.8	-	1	-	1	36	8*^{2}	-	-	19.6

* Represents embryos in which 2 good analyzable cells were found to have 39 chromosomes.

According to this hypothesis, trisomy is predetermined by the order of formation of oocytes during fetal ovarian development, late-formed oocytes with fewer chiasmata and more univalent pairs being the last to be ovulated. These, it is argued, could contribute univalent chromosomes which might segregate randomly to the poles at anaphase 1 of meiosis and produce aneuploid oocytes in aged females. Certainly, declining chiasma frequencies and increasing univalent frequencies have been found in aging mouse oocytes by a number of authors who have studied the problem (14,19, 24,27). Neither Polani and Jagiello (24) nor Speed (27), however, could confirm the expected association between an increased occurrence of univalents at metaphase 1 in the mouse, and hyperploidy at metaphase 2. More precise findings were made for the Chinese hamster by Sugawara and Mikamo (31). In that species, the meiotic chromosomes can be divided into 4 morphologically distinguishable subgroups and so it is possible to determine whether the same chromosome pair that forms univalents at M1 actually undergoes nondisjunction at the first meiotic division. Sugawara and Mikamo, in fact, found no correlation. The incidence of M1 oocytes with a univalent pair and of aneuploid M2 oocytes due to first-meiotic nondisjunction was significantly higher in aged females, but univalents occurred almost exclusively in the smallest metacentric chromosome group, whereas nondisjunction took place nearly equally in all chromosomal subgroups. These authors concluded that a relationship between univalent formation and nondisjunction, the basic tenet of the production line hypothesis, had been disproved by their experiments, and suggested that some factor operating in the adult female was more likely to be the direct cause of age-related aneuploidy in oocytes. Spindle defects were proposed (31).

Before leaving the subject of the production line hypothesis, it is worth pointing out that cytological studies carried out by Speed and Chandley (29), employing spreading methods for fetal oocyte preparation, failed to reveal evidence for increased univalent incidence with advancing gestational time in the mouse. Neither did other synaptic anomalies, which might in turn have contributed to a lowering of chiasma frequencies, occur with greater frequency among late-formed oocytes. The existence of a production line in the mouse, therefore, remains questionable.

Human fetal oocytes, by comparison with the mouse, show some extremely interesting anomalies. Another recent study employing spreading techniques, also carried out by Speed (28), reported on the progression of meiotic prophase in human oocytes from a series of aborted fetuses spanning the first 2 trimesters of gestation. The pachytene stage, which was first seen at 16 weeks gestation, was examined in detail at the electron microscopic level in a search for evidence of synaptic anomalies. A total of 162 oocytes in pachytene from 6 fetuses were analyzed over weeks 16-23, and a suprisingly high frequency of synaptic anomalies was found. Altogether, about 20% of oocytes were affected by such anomalies. They ranged from single or double sets of univalents, partial asynapsis in one or more (up to a maximum of 7) bivalents, and occasional oocytes in which almost the entire genome appeared to have failed in synapsis at the zygotene stage. Among oocytes showing partial asynapsis in a single bivalent, the degree to which pairing failure was expressed varied from 5% to 50% of total axial length, the unpaired axes showing marked thickening and dark staining with silver. In some cases, excrescences were present resembling, in appearance, those frequently observed along the differential axes of the X and Y in human spermatocytes. The significance of such thickenings and excrescences remains obscure, but they seem to characterize asynaptic axes, and it may well be that pairing failure early in zygotene, or lack of pairing attributable to lack of a pairing partner (as in the case of the X and Y differential axes), leads to an overproduction of some proteinaceous material which otherwise would be limited in amount and controlled by the act of pairing itself. The hypothesis that there are meiotic pairing sites which must be "saturated" by homologous pairing (21) is one which could be considered in this context. In unpaired regions along the chromosome axes, these sites might be overactivated, thus causing excessive production of a protein leading to the thickening of axes and production of excrescences.

It has been suggested (5,21) that the failure to saturate pairing sites could set in motion a process which eventually leads to the destruction of oocytes and spermatocytes. Were this to be the case in the human fetal ovary, oocytes carrying univalent pairs would be selected against in the germline, and would not constitute a threat of aneuploidy production by the adult female (14). It is nevertheless interesting that when comparisons are made between pachytene oocytes of the mouse and those of man, about 10 times more errors of synapsis leading to univalent pairs are seen in the latter species (28). Similarly, aneuploid conceptions are known to be more common by at least an order of magnitude in man than in the mouse (10), and of course, the majority of human errors are known to arise at meiosis in the female (12). A contribution from oocytes containing univalent chromosomes to the overall high levels of nondisjunction in man cannot, therefore, at this stage, be ruled out. Nevertheless, the overall levels of such preformed synaptic anomalies still remain small by comparison with the predicted high levels of nondisjunction occurring in human oocytes, and particularly in those being ovulated late in life (see earlier elucidation). To explain the data adequately, we need to be looking for some factor that can lead to aberrant meiotic chromosome segregation, not just in 10% or 20% of oocytes, but in the vast majority of them at late reproductive age. We now believe we may have identified such a factor.

From the beginning, one etiological factor associated with aging in the ovary or oocyte that has been propounded more than any other has been spindle malformation or malfunction (1,23,31). The demonstrations that meiotic nondisjunction can be associated with a defect of spindle microtubules in oocytes having undergone intrafollicular preovulatory overripeness (6,20), or those having been treated with spindle poisons such as colchicine (30), add weight to this argument. Inadequate tubulin polymerization, which causes incomplete formation of spindle microtubules, has been suggested as the direct cause of the increased incidence of meiotic chromosome nondisjunction in the oocytes of aged females of the Chinese hamster (31).

The search for direct evidence of spindle abnormality associated with natural aging in mammalian eggs has never previously been undertaken, but is now underway in our laboratory. Using the mouse as a model, Dr. Ursula Eichenlaub-Ritter has developed techniques for the preparation and study of spindle structure in CBA oocytes at different ages of the reproductive span. Her work is still at an early stage, but initial results leave no doubt that a structurally altered spindle apparatus, combined with displacement of chromosomes from the metaphase plate, is indeed a very common feature of aged oocytes. The frequency with which such anomalies occur in the oocytes of old CBA females compared with young gives us real encouragement in the belief that we may have hit on an extremely important age-associated defect and one which could underlie the age effect for aneuploidy not only in the mouse, but also, by extrapolation, in man.

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DISCUSSION

HOOK: I wonder if Dr. Chandley would again state her hypothesis as to the views for why aging is associated with increased frequency of trisomy? I wonder if you would also comment in response to Dr. Hansmann's comments about the nonextrapolability or the implicit nonutility of the mouse for extrapolating to humans compared to other species? I believe you said it was CBA mice that were the most useful. In a book by Bond and Chandley (1983) I noted at least 2 experiments with CBA strains, including one by Golbus (1981), in which he had, in young mice, 17 out of 258 hyperploid oocytes, and in old mice, 8 out of 217. It diminished in aging. Would you elaborate on this?

CHANDLEY: I would suggest that seeing increasing numbers of spindle abnormalities with aging gives us some clue that this is bound up with an increase in trisomy. I am certainly not the first to suggest that spindle abnormalities could relate to increase in aneuploidy and nondisjunction, but it is very nice to see that there really are spindle abnormalities developing with aging. This, to my knowledge, has never been demonstrated before. We do not know what causes the spindle abnormalities; that is the next thing we will have to find out. It is obviously an epiphenomenon of the aging process in the female. In the mouse, I agree, it is difficult because we do not get the dramatic increases in aneuploidy with aging that we do in humans. In the mouse, some studies, as you say, do not show age-related increases (e.g., Golbus [1981], etc.), but there are others that do (e.g., Gordon [1973], Fabricant and Schneider [1978], Brook et al. [1984]). Dr. Eichenlaub-Ritter is seeing a lot of spindle abnormalities (up to 60% among aged oocytes in CBA mice), but we do not see such a great rise in aneuploidy (Brook et al. [1984]). We have yet to reconcile this. Maybe the mouse has more ability to repair or restore its spindle abnormalities, or at least to prevent them from causing nondisjunction. Human spindles have not yet been studied. Maybe there is some special feature of the human oocyte spindle that leads more to nondisjunction.

HOOK: You mentioned the difference between chronological and biological aging earlier. These inferences were tied to the spindle abnormalities by virtue of the observation that when you ovariectomized mice, you saw earlier increases in both aneuploidy and spindle defects. Would you comment on that once again?

CHANDLEY: Dr. Eichenlaub-Ritter has not had time yet to look at many oocyte spindles from unilateral ovariectomized mice. But the ones she has looked at show the same kinds of shrinkage and displacement of chromosomes seen naturally in aged oocytes, but at an earlier age than in controls (sham operated) and at the age when we know the aneuploidy starts to arise in those animals (Brook et al. [1984]). So a correlation seems to exist.

HANSMANN: We tried very hard during the last 5 years to measure any effect of maternal age on nondisjunction in mouse oocytes. We investigated also biological age, but from another point of view. We irradiated young females, 1-week old, with very low doses of X-rays, which are known to decrease the number of primary follicles. We analyzed spontaneously ovulated oocytes at ages of 10, 20, 30, 40, 50, and 60 weeks, to pick up, if it does happen, a hump-shaped curve of nondisjunction with age as shown by Martin and colleagues in 1976. We also made measurements using radioimmunoassay hormone levels. This irradiation at very young ages does in fact cause premature reproductive failures. That means the number of ovulated oocytes decreased 20 weeks earlier. The endocrinological profile typically alters 20 weeks earlier, but we only observed 1% anucleated oocytes in the 50-week-old age group. But this was not a significant increase at all compared to all age groups. We also made histological sections through the ovary in a different strain. At high ages, the ovary was depleted of all oocytes. So, significantly, it could be compared to the human situation or to CBA. We were puzzled about these results after working so long on this. One possibility which most of these groups have not considered is that in these experiments on age, usually all females or groups of females are caged together in groups of 10 or 15, depending on the size of the cage. It is known that groups of females have one female which inhibits estrogen cycles in the other females in the same cage. So it could be that these females kept in groups up to the age of analysis do not correspond to the normal situation of individual females allowed to age up to this same number of weeks. So they may be older in age, but possibly they have not had the chance to have cycles up to this age, and to deplete the ovary. It could be that such errors may be due to the failure to detect consistently in all these studies an increase of nondisjunction with age in the mouse.

FORD: When we wanted to look at the question of aneuploidy in humans, we decided not to go around with large biological hammers and smash the cells to pieces, but to try and mimic the sorts of changes that might happen in vivo. I read my MT and MT-inhibitor book from the Janssen Pharmaceutica Conference, and found, at least in vitro, that MTs that were polymerized under conditions of unfavorable pH--that is, just outside the physiological range--would be shorter and would tend to be disoriented from assembly centers. A similar thing is found with raised calcium. I thought that this was an interesting model to choose, so I played around with this. Not only did I find effects on displacement in aneuploidy, but also, when I looked at my spindles using fluorescent tubulin (antitubulin) in the same way as Dr. Chandley, I saw the same sorts of spindles. I can very much support that her studies are, in fact, the sorts of things you see if you change your physiology subtly. We have also done some EM studies on this. They are only preliminary at the moment, but they also give you the idea that under subtle changes of pH, you have a sort of jiggly metaphase. Your plate is just not as well aligned (it is probably shorter) and the MTs tend to be disorientated.

MECHANISMS OF NONDISJUNCTION: FACTS AND PERSPECTIVES

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SUMMARY

An ordered segregation requires distinct processes of differentiation within the germ cell for recognition and segregation of homologous chromosomes/chromatids. These include synchronous maturation of the nucleus and cytoplasm, chromosome pairing and assembly at the metaphase plate, and movement within the spindle; all of them may be under direct/indirect regulatory control by the surrounding somatic compartments. Interference, e.g., by hormonal alterations at any of these steps, may alter the normal program of differentiation, thus increasing the risk of chromosomal malsegregation. Hence, many different causative mechanisms may exist which basically, nevertheless, act via (a) nonsegregation, (b) chance segregation, both during meiosis 1 and 2, or (c) presegregation during meiosis 1.

In our animal models of the Djungarian hamster and the NMRI/Han mouse strain, we are analyzing the mechanisms of nondisjunction and presegregation during meiosis 1 in oocytes, as well as during aging of the females, by the application of hormones (gonadotrophins and steroids) and specific microtubular inhibitors (colchicine and methylbenzimidazolcarbamate). We suggest that chromosomes play a relatively passive role, although chromosomal properties, e.g., length, chiasma number, NORs, and position within the spindle, may provide an individual risk for each bivalent to be affected by nondisjunction. Failures in the endocrine control of follicular and germ cell maturation are considered to be primary causes for nondisjunction in young and aging oocytes. The understanding of the differentiation processes resulting in the maturation of follicles "at risk" may provide us with the tool to prevent the generation of aneuploidy in man.

INTRODUCTION

The chromosomal nature of autosomal and gonosomal human disorders was described 26 years ago by cytogeneticists (17,34,35,39), and hence, most hypotheses, like spindle degeneration (1,42), nucleolus persistence (14,46), or the "production line" (29), are only based on morphological/-mechanical criteria and explain the association of trisomy with maternal age (see Ref. 7 and 19 for review).

Aspects of gonadal function and maturation of germ cells within and in coordination with the somatic compartment of the gonad in young and elderly individuals have been neglected so far. Germ cells, however, are physically and metabolically coupled with the surrounding somatic cells during most stages of meiosis (13). Despite the potential capability of chemical and physical, as well as other, mutagens to induce aneuploidy in man (7,19, 50), it is apt to assume that endogenous causes are responsible for most errors of chromosome segregation during human meiosis. Based on the results from our studies with ovulated oocytes from Djungarian hamsters and NMRI/Han mice we proposed recently that "failures of the endocrine control of meiosis" cause an altered developmental program within the maturing germ cell, thus increasing the risk of aneuploidy (19,26). Such failures of differentiation may lead either to nondisjunction, chance segregation (both events may occur at meiosis 1 and 2), or presegregation during meiosis 1 (21,47). So far it is unknown which of the events is biologically most relevant in man; yet we assume that nondisjunction during meiosis 1 frequently causes aneuploidy in mammalian oocytes. Model systems are required for studying the different events individually (e.g., Type I, A-C; Type II, A-B [19]), to describe processes preceding aneuploidy in biochemical and molecular terms.

ANEUPLOIDY BY FAILURES OF ENDOCRINE CONTROL

Studies with Djungarian Hamsters (Phodopus Sungorus)

Influence of gonadotrophins. During the course of our cytogenetic studies on the incidence of spontaneous chromosome anomalies in oocytes and early cleavage stages from various mammalian species (21,22,23,49), we noticed that gonadotrophins caused the ovulation of significant numbers of aneuploid oocytes in Djungarian hamsters (25). The effect of induced aneuploidy has been constantly present in females from our breeding colony during the past 7 years. The colony is derived from 5 breeding pairs, originally obtained from Dr. Kurt Bürki during his stay at the Cancer Research Institute, Lausanne; inbreeding has been avoided during all these years. The gonadotrophins used are pregnant mare serum (PMS; Intergonan, Vemie Chemie, Kempen; Federal Republic of Germany) and human chorionic gonadotrophin (HCG; Organon, München; Federal Republic of Germany), both dissolved in Ringers solution and injected intraperitoneally 48 hr apart from each other. After that hormonal pretreatment approximately 70-90% of all females respond and ovulate, the number of ovulated oocytes dependent on the doses of gonadotrophins injected (6-12 oocytes as a mean). Littersize after spontaneous ovulation is around 5½. The karyotype (2n=28) is very suitable for cytogenetic analysis and chromosomes can be identified individually in, for example, ovulated oocytes without specific banding techniques (Fig. 1).

Two types of aneuploidy are generated by gonadotrophin treatment: hyperhaploid (Fig. 1) and diploid (Fig. 2) oocytes (25). Hypohaploid metaphases, which can be at least partly due to preparational loss, are only rarely observed. We assume, therefore, that the type of gonadotrophin-induced chromosomal malsegregation is not only due to real nondisjunction, but that the affected bivalents remain also preferentially within the oocyte and are only exceptionally extruded into the first polar body. Several types of diploid oocytes are observed (Fig. 2). Some are arrested at diakinesis/metaphase 1 (Fig. 2a). Few are blocked during bivalent segregation and show various transitional stages between metaphase 1 and anaphase 1 (Fig. 2); others have a diploid metaphase 2 configuration (Fig. 2d).

The frequencies of both types of aneuploid oocytes depend on the doses of gonadotrophins injected, being around 1% at low doses and reaching

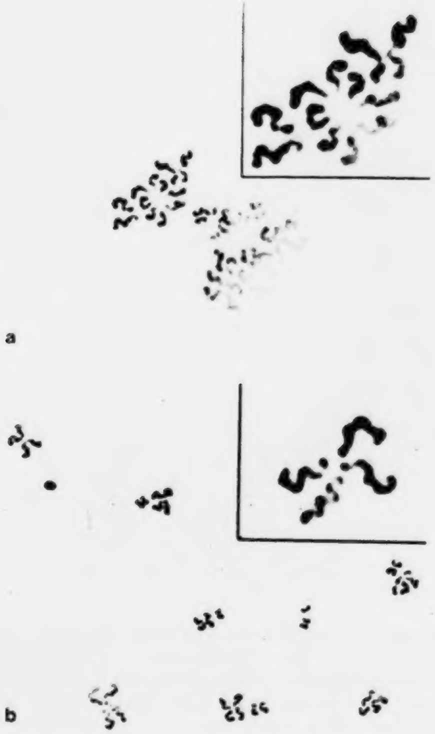

Fig. 1. Examples of hyperhaploid metaphase 2 oocytes from Djungarian hamsters (prepared by F. Beermann). (a) Additional A-group chromosomes (n+1=15). The characteristic association, commonly observed, of the affected bivalent is shown in the inset. (b) Additional D-group chromosome (n+1=15). Both chromosomes of the affected bivalent are still associated with their NORs (inset). The NOR on this chromosome 5 is located distally on the short arm (6). This is a first example in germ cells that the chromosomal property, "nucleolus-organizer," is involved in nondisjunction, at least in the hypergonadotrophic condition.

approximately 10% after 10 i.U. (international units) each of PMS and HCG. Surprisingly, several bivalents may be affected by nondisjunction in the same oocyte; metaphases with 2, 3, or even more odd chromosomes (up to 6 surplus chromosomes) are observed. Combinations with various doses of gonadotrophins revealed that both gonadotrophins, PMS and HCG, are effective and also required for hormone-induced aneuploidy. These gonadotrophins most likely do not act directly in or onto the oocyte since no specific receptors on oocytes have been identified thus far (53), but their effects seem to be mediated in a so far unknown manner via the somatic compartment, via theca and granulosa cells. The last 48 hr of follicular differentiation appear to be crucial to create a situation which may generate aneuploidy, according to our experiments with various dosage combinations of gonadotrophins.

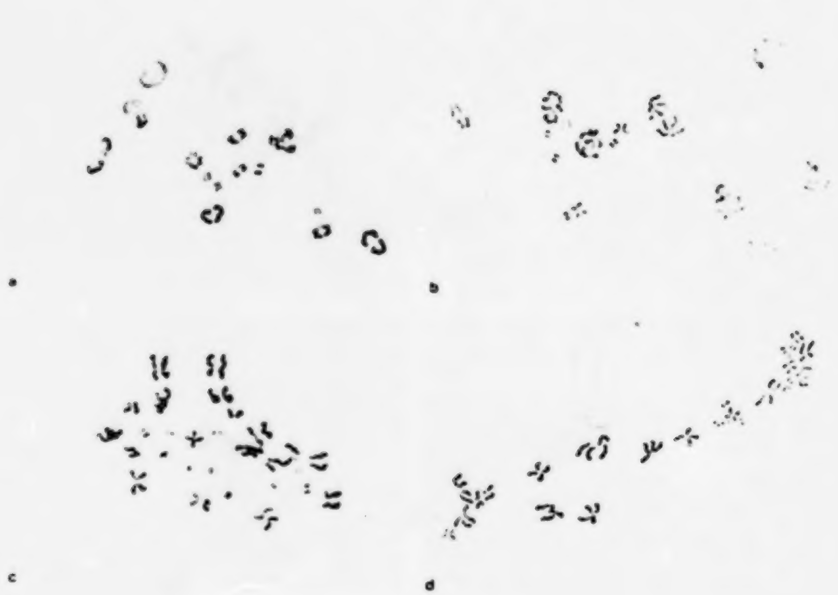

Fig. 2. Examples of ovulated diploid oocytes from Djungarian hamsters (prepared by F. Beermann). (a) Arrested at metaphase 1. (b) Transitional stage between metaphase 1 and anaphase 1. Chromosome segregation has just begun; some bivalents are still associated with each other. (c) Advanced transitional stage (MI to AI). Most have separated. (Note that both D-group chromosomes are orientated with the NORs towards each other.) (d) Diploid metaphase 2 configuration; all bivalents have separated from each other.

Recent experiments with gonadotrophin application to prepubertal females (51,52) corroborate our initial observations. For this reason we conclude that aneuploid oocytes in adult females are not derived from atretic or degenerated follicles matured during previous cycles. In addition, according to measurements, hyperhaploid oocytes also have the normal size of oocytes in fully grown follicles and that of normal oocytes after ovulation (52). Nevertheless, according to histological studies of follicular maturation in prepubertal females, specific types of follicles seem to be at risk to ovulate preferentially aneuploid oocytes (51).

The analysis of more than 100 events of nondisjunction revealed that not all bivalents have the same chance to be involved in nondisjunction during the first meiotic division (4,25,51). Large meta- and submetacentric chromosomes from groups A-D are most often affected. Chromosomal properties, such as length (which may be associated with number of chiasmata), existence and position of a nucleolus-organizer, and position of a bivalent within the spindle, provide individual risks for each bivalent to be involved in nondisjunction in the hypergonadotrophic situation (19).

As a hypothesis to work with (19,20,26) we proposed that an altered follicular maturation causes a more or less asynchronous maturation of the nucleus (e.g., from germinal-vesicle breakdown onwards) and the cytoplasm in the oocyte, which finally results in the premature inhibition of single/multiple (hyperhaploid), or even all (diploid, metaphase 1) pole-to-kinetochore microtubules or pole-to-pole microtubules (diploid, e.g., metaphase 2 configuration). We further suggested an asynchronous segregation of bivalents during anaphase 1 (see also Fig. 2).

Effects of Spindle Inhibitors on Chromosome Segregation

(a) Colchicine. To verify the concept of the asynchronous segregation during anaphase 1 as a cause for preferential nondisjunction, the influence of colchicine on the first meiotic division was investigated (32). This spindle poison binds specifically to tubulin (2,37) and inhibits the polymerization of microtubules (40). After initial experiments to stage the progress of meiosis during the preovulatory phase from germinal-vesicle breakdown onwards, colchicine (3 mg/kg body weight) was applied to adult Djungarian hamsters, primed with low doses of gonadotrophins, at a rather late stage of oocyte maturation (5½ hr after HCG-injection), which, however, allows treatment before anaphase 1. Such late colchicine application resulted in the ovulation of significant numbers of diploid (34.5%) and hyperhaploid (11.7%) oocytes. And indeed, the same bivalents as in the hypergonadotrophic situation were affected by nondisjunction, namely the large meta- and submetacentric chromosomes from groups A-C. Thus, a late inhibition of microtubular function increases the likelihood for nondisjunction specifically for "late segregators." Surprisingly, chromosome 5 (group D), which carries a nucleolus organizer distally on the short arm (6), was never observed in colchicine-induced hyperhaploidy. This bivalent, however, was very often involved (see also Fig. 1b) in nondisjunction following stimulation with high gonadotrophin doses (25,51). Thus, nucleolus persistence, as suggested by Polani et al. (46) and Evans (14), may be a chromosomal property predisposing to nondisjunction. Further studies will investigate whether a hypergonadotrophic situation enhances rDNA transcription or affects the transport of rRNA from the transcription site, or whether nucleolus-associated proteins accumulate. Presegregation, which occurs in less than 1% of control oocytes, was not significantly increased after late-colchicine treatment (1.5%) (32).

(b) Methylbenzimidazolcarbamate. The benzimidazol-derivative, methylbenzimidacolcarbamate (MBC), is considered to be responsible for the antimitotic activity of benomyl (10). In Aspergillus nidulans it binds to a 110 kd protein (11); the β-subunit of tubulin is considered to be the binding-site for benomyl (43). Hoebeke et al. (30) suggested that both colchicine and derivatives of benzimidazole have affinity for the same binding site. The compound MBC was used, therefore, to investigate the question of whether the observed pattern of nondisjunction of specific bivalents ("late segregators") after colchicine and high doses of gonadotrophins is characteristic for the inhibition of microtubular function only at a late phase during first meiotic division.

MBC (1,000 mg/kg body weight) caused, in contrast to colchicine, the ovulation of preferentially hyperhaploid (20-40%, depending on the interval after HCG-injection), and less often of diploid, oocytes (31). The late application of MBC (6 hr after HCG) gave results according to the studies mentioned previously, with specific nondisjunction of predominantly single bivalents and again from the large meta- and submetacentric chromosomes.

Up to 7 bivalents (n+7) were affected by nondisjunction in individual oocytes after an MBC-injection earlier during the preovulatory phase (4½ hr after HCG; 1,000 mg/kg body weight). The pattern of malsegregated bivalents was rather different from the pattern observed after inhibiting the late spindle. After that late inhibition all bivalents participate in nondisjunction at frequencies which can be calculated as probabilities based on the relative length of each individual chromosome at metaphase (calculated from bone-marrow metaphases).

However, after early inhibition of spindle-function by MBC each bivalent seems to have the same chance to be involved. Observed frequencies for individual chromosomes differ significantly from expected probabilities calculated on the basis of chromosome length, but they do not differ significantly from expected frequency when each bivalent has the same chance.

Preliminary data with oocytes from Chinese hamsters (Hansmann et al., unpubl. observ.) corroborate the observation that inhibition during late phases of spindle function causes nondisjunction of "late segregators." Hence, our conclusions with respect to the individual risk of bivalents for nondisjunction and the dependence on the stage when spindle function is inhibited appear to be of more general validity.

Studies with the NMRI/Han Mouse

Influence of gonadotrophins. NMRI/Han mice spontaneously ovulate diploid oocytes (less than 1%) which are most often (as in oocytes from Djungarian hamsters) arrested at metaphase 1 (21). Their frequency increases also with the dosage of injected gonadotrophins. In contrast to the situation in Djungarian hamsters, we never observed significantly more hyperhaploid oocytes, suggesting that the failure to detect nondisjunction, despite the present sensitivity to gonadotrophins in that strain, may originate from a species-specific, different pattern of chromosome segregation during anaphase 1 (19,20). The mouse, accordingly, is believed to represent a species with a more or less synchronous segregation of all bivalents during anaphase 1 (Fig. 3).

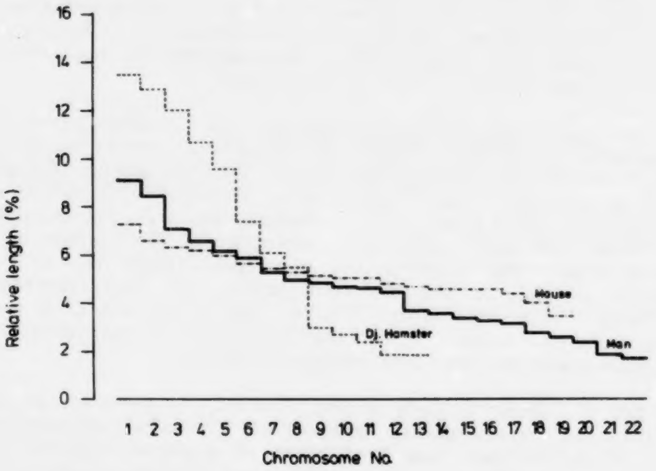

Fig. 3. Comparison of karyotypes from mouse (44), man (45), and Djungarian hamster (31) with respect to the heterogeneity. When chromosome length at mitosis (or that of the synaptonemal complex) is one of several possible predeterminations (e.g., NORs, C-heterochromatin) for asynchronous/synchronous segregation at meiosis, then those species with a pattern like the Djungarian hamster would be more sensitive for induced nondisjunction compared to species like mouse. The human karyotype seems to represent an intermediate position.

Hormonal alterations during late follicular development either allow the ovulation of normal haploid oocytes or, in extreme situations, block the maturation before the 1st meiotic division. The Djungarian hamster seems to be one example of a species with a rather asynchronous segregation during anaphase 1 (Fig. 3), and may be more suitable for mutagenicity studies than the mouse. The irradiation of gonadotrophin-primed NMRI/Han females during an early preovulatory phase with rather low X-ray doses surprisingly decreased the incidence of ovulated diploid oocytes (26). Such a protective effect of irradiation was considered to be caused neither by selective killing of diploid oocytes nor by compensatory superovulation of normal oocytes, preferentially. The effect of irradiation was explained by an adverse influence on the "asynchronous hypergonadotrophic maturation" which reconstituted a more or less synchronous pattern within the oocyte. This example demonstrates that results from mutagenicity studies may provide information on such basic events of proliferation of germ cells.

Cytogenetic studies with ovulated oocytes from 2 other mouse strains and their F_1-hybrids from crosses with the NMRI/Han strain provided some more information useful for understanding the mechanisms underlying nondisjunction (20). The 2 other mouse strains, C57BL and BALB/c, were chosen because of their different H2-type and their known different pattern of cleavage during early preimplantation development. Embryos from the C57BL strain develop more rapidly than, for example, those from BALB/c and NMRI/Han. Adult females from C57BL and BALB/c are "nonsensitive" to gonadotrophins and never ovulate diploid or hyperhaploid oocytes. Surprisingly, F_1-hybrid females from crosses with NMRI ovulated, after gonadotrophin-priming, a significantly increased frequency of hyperhaploid oocytes. We concluded that genetic factors responsible for the endocrine control of follicular maturation and those for cell proliferation per se are indeed important, and that heterozygosity for genes of cell proliferation may influence the ordered segregation of chromosomes during meiosis. Further studies will have to show whether oncogenes, and if so, which ones, are expressed in germ cells and may thus also be involved in chromosome segregation.

To study the inheritance of the trait, "gonadotrophin-induced diploidy," we analyzed oocytes from the parental strains, NMRI, C3H, BALB/c, and C57BL, as well as from F_1-hybrids (3,36). Females from F_1-hybrids and also from back-crosses to NMRI-males (in breedings with C3H) ovulated significant numbers of diploid oocytes. Hence, the sensitivity for gonadotrophin-induced diploidy, at least in mice, is genetically determined and the trait expressed in oocytes is transmitted not only maternally, but also paternally (3). Another observation which may help us to understand the underlying basic mechanisms of an ordered chromosome segregation was made during these breeding experiments. The frequencies of gonadotrophin-induced diploidy in ovulated oocytes from the various crosses are indicative of an apparent maternal influence which may modulate the expression of the trait, "diploidy." In, for example, breedings between NMRI/Han and C57BL, those F_1-females were most sensitive which came from crosses between NMRI-males and C57BL-females, which themselves do not respond to gonadotrophins with the ovulation of diploid oocytes. The observed modulating maternal factor may result from an interaction between the maternal environment and the embryo during gestation, or even after birth up to weaning; however, the factor may be inherent to the oocyte as well. A recent example of such a maternal inheritance is given by Fischer-Lindahl et al. (16). Mitochondria are exclusively of maternal inheritance; they synthesize ATP, and the system for side-chain cleavage of cholesterol to pregnenolone, as a rate-limiting step in steroidgenesis, is located within mitochondria (38,48). We investigated the role of mitochondria, affecting their function during the late phase of follicular maturation by treatment with chloramphenicol, a potent inhibitor of mitochondrial peptidyl transferase. Our first results revealed that chloramphenicol not only inhibited the characteristic increase of ovarian weight and the increase of progesterone concentration within the postovulatory ovary, but it also increased concomitantly the incidence of ovulated diploid oocytes (5). Yet, only studies with congenic strains differing in the type of mitochondria will clarify the role of mitochondria in the generation of gonadotrophin-induced aneuploidy.

The model system of gonadotrophin-induced diploidy in oocytes from NMRI/Han mice proved to be essential in testing, in addition to the studies on inheritance, another basic assumption, namely, that of the control of meiosis by the surrounding somatic compartment. To study the fate of "sensitive" NMRI/Han oocytes in a "resistant" somatic environment and vice versa, we analyzed the incidence of diploid ovulated oocytes after gonadotrophin-stimulation in NMRI/Han ↔ C57BL chimeric females. The origin of the ovulated oocytes was traced by the presence or absence of marker chromosomes (T1G8 in NMRI/Han). All "sensitive" oocytes ovulated from chimeras proved to be normal haploid (3). Our still preliminary data, indeed, suggest that oocyte maturation and chromosome segregation are controlled by somatic cells.

MATERNAL AGE AND ANEUPLOIDY

Studies with Ovulated Oocytes from Djungarian Hamsters

A number of cytogenetic studies have been performed with pre- or postimplantation embryos and ovulated oocytes from aging mouse and Chinese hamster females, with somehow contradictory results (for review, see Ref. 19). Golbus (18), focusing mainly on altered survival of aneuploid conceptuses as the cause for increase of trisomies with maternal age, even neglects the existence of age-dependent nondisjunction in mammalian oocytes.

Investigating the significance of our Djungarian hamster system to the study of maternal age-dependent processes of chromosome segregation, we cytogenetically analyzed ovulated oocytes derived from females of 3 different age groups: young (8-14 weeks), middle-aged (26-46 weeks), and old (49-75 weeks) (33). Ovulated oocytes were analyzed at the metaphase 2 stage to avoid any interference of secondary effects, during and after fertilization, with the primary frequency of genome mutations at meiosis 1. Furthermore, this procedure made possible a detailed analysis of the different events (presegregation versus nondisjunction) causing trisomy. Three aneuploidy types were observed in oocytes from aging females, namely diploid and hyperhaploid oocytes, as well as oocytes showing presegregation (metaphases with a missing or surplus chromatid). The frequency of hyperhaploidy was significantly increased in aged oocytes and reached 3.6% in females from the oldest group. The frequency of bivalents affected by nondisjunction was 3.97×10^{-4} in young females and 3.97×10^{-3} ($P < 0.05$) in the group of middle-aged females. However, there was no further increase in the number of involved bivalents at higher ages.

Altogether more than 20 individual chromosomes were identified as being involved in nondisjunction in the middle-age group, and 3/4 of them belong to the group (A-D) of large meta- and submetacentric chromosomes. Hence, the maternal age-dependent processes of nondisjunction cause a pattern similar to that after treatment with microtubule-inhibitors and after hypergonadotrophic treatment. The basic mechanisms inhibiting microtubules at the late phase of meiosis 1 spindle function may be similar in all 3 experimental conditions. Surprisingly, no specific pattern of involved bivalents was detected in oocytes from the very old females, an observation which could also be due, however, to the smaller sample size in that group.

Presegregation occurred slightly more often in oocytes from aging Djungarian hamster females, yet the difference was not statistically significant. No specific chromosome seemed to be preferentially involved in presegregation. Polani and Jagiello (47) and de Boer and van der Hoeven (12) found, accordingly, a slight increase of presegregation in oocytes from mouse females at ages 40-89 weeks and 48-60 weeks, respectively (for review, see Ref. 19). Thus, presegregation occurs in oocytes from different species regardless of whether they have only acrocentric chromosomes, or all types of other chromosomes (22). Hence, presegregation may be a significant contribution to trisomic conceptuses in man also, and its frequency may increase, even if only slightly, with maternal age. The frequency of ovulated diploid oocytes decreased with females' ages, which is in apparent contrast to the observations made on the aforementioned events. Either diploidy and hyperhaploidy just represent alternating phenomena with only one identical cause or the events resulting in diploidy/hyperhaploidy are actually distinct from each other.

Studies with Ovulated Oocytes from NMRI/Han Mice

In analyzing cytogenetic data from prenatal diagnosis after amniocentesis, Ferguson-Smith (15) suggested a leveling-off, or even a decrease, in the slope of at least certain trisomies at very advanced maternal ages. Accordingly, such a hump-shaped slope for the frequency of hyperhaploid oocytes was observed in the CBA mouse strain (41). Martin et al. (41) analyzed oocytes from 3 groups of females (8-21, 21-34, and 34-47 weeks) and found 6 hyperhaploid metaphase 2 oocytes among 95 analyzed from the middle-aged females. Neither in young nor old females were any other hyperhaploid metaphase-2 oocytes found. To study the possibility of a hump-shaped slope for maternal age-dependent nondisjunction we analyzed the chromosomes from oocytes spontaneously ovulated from NMRI/Han mice at ages 10, 20, 30, 40, 50, and 60 weeks (24). Hyperhaploid oocytes were only detected in the group of 50-week-old females (2.8%), none at younger or older (60-week) ages. The frequency of spontaneously ovulated diploid oocytes decreased to a statistically significant degree in females from age groups 10 to 40 weeks, an observation which is in agreement with the above mentioned data from Djungarian hamsters. However, diploidy increased again at higher ages in NMRI mice.

Our data with the NMRI/Han strain show that nondisjunction during meiosis 1 does not continously increase with the female's age. Several reasons may account for this unexpected observation. One explanation could be that animal maintenance (approximately 15 females were kept in one cage in our experiments) up to the respective age at preparation causes some artificial condition in which the females are not allowed to age properly. It is rather speculative to assume that conditions causing nondisjunction in dependence of maternal age are correlated in some way with cyclicity of ovarian function, whether with irregular cycles or not. In addition, anestrous behavior is commonly observed in mouse strains after caging many females together. Another explanation may be found with the karyotype of the mouse. The mouse has only acrocentric chromosomes which are similar in size (Fig. 3), and, as mentioned before, we guess that all bivalents segregate synchronously during meiosis 1 in this species. Furthermore, spindle function may be different in the mouse, and/or inhibitors (those which arrest oocyte progression at metaphase 2) may have different affinities, for example, for binding preferentially to pole-to-pole microtubules, thereby causing the ovulation of diploid oocytes.

Biological Versus Chronological Age and Aneuploidy

Brook and colleagues (9) unilaterally ovariectomized 6- to 8-week-old CBA mice and maintained these, together with sham-operated females, in groups up to the ages of 63-91, 154-182, and 245-280 days. After having reached the given ages, they were mated with young males and their preimplantation embryos prepared cytogenetically on day 4 and analyzed for numerical chromosome anomalies. They observed hyperdiploid, triploid, and tetraploid embryos and claimed, based on rather small numbers of typed embryos, that the maternal age-dependent increase of aneuploidy did occur earlier in unilaterally ovariectomized females. This kind of ovariectomy caused fewer numbers of estrous cycles and an earlier onset of acyclicity. According to our own observations on that aspect (Tab. 1) (24) and also according to our concept of "endocrinological alterations as causes for aneuploidy" (25), we principally agree with the suggestion from Brook and colleagues (9). Apparent differences in the results between both studies may be due to either strain specificity, a different efficiency of both methods to promote biological aging, or the fact that by analyzing embryos the occurrence of meiosis 2 and mitotic errors are also depicted.

Four years ago we started our experiment with 2 groups of NMRI/Han females and analyzed body and ovarian weight, ovulation behavior (after mating with vasectomized males), number of ovulated oocytes, and the incidence of hyperhaploid and diploid oocytes at ages of 10, 20, 30, 40, 50, and 60 weeks (± 2 weeks at each group). Furthermore, concentrations of the steroids progesterone, estradiol, and testosterone were measured by radioimmunoassay in females of the respective age groups. Our strategy was not to remove one ovary, but to decrease the total pool of nongrowing follicles in both ovaries by irradiating young, prepubertal mice with low X-ray doses, known to cause degeneration of oocytes. Thus, 7-day-old females received 0.05 Gy (Gray) in the experimental, and 0.0 Gy in the control group.

Females from the irradiated group showed, precociously, irregularities in ovulation behavior, i.e., the number of females with vaginal plugs but without ovulated oocytes increased earlier, and the number of ovulated oocytes soon dropped drastically. Few oocytes were obtained from 50-week-old females and none from females at 60 weeks. The measured patterns of ovarian steroids were drastically altered. In the control group the ratio

Tab. 1. Chronological versus biological maternal age and the generation of aneuploidy.

Group	Strain	Analysis of	Treatment with	% aneuploid oocytes/embryo (n analyzed) from mouse females at ages (weeks) of					
				8-12	18-22	28-32	38-42	48-52	58-62
Hansmann et al. (26)	NMRI/Han	oocytes	control	1.6 (506)	0.8 (479)	0.8 (883)	0.2 (569)	3.1 (572)	1.0 (580)
			X-rays (0.05 Gy)	0.3 (301)	1.0 (275)	0.0 (302)	1.3 (288)	3.0 (33)	--
				9-13	22-26	35-40	44-50		
Brook et al. (9)	CBA/CA	embryos	control	1.5 (66)*	0.0 (65)	3.5 (58)	4.4 (46)		
			ovariectomy (unilateral)	0.0 (67)	6.2 (65)	13.9 (36)	--		

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* Only diploid, hyperdiploid, and polyploid embryos are included in the calculation of aneuploidy, due to the possible artificial loss during preparation as cause for hypodiploidy.

of testosterone/estradiol decreased from approximately 2.5 at 10 weeks to 0.5 at 60 weeks. Females from the experimental group, however, proved to have completely different concentrations. The ratio increased, instead, from approximately 3.5 at an age of 10 weeks to 6.0 at 50 weeks. Concomitantly, an apparent increase in progesterone concentrations was observed. Aneuploid oocytes appeared in nearly all age groups of controls and irradiated females (Tab. 1). The data are based on large numbers of analyzed oocytes in each group ranging from 288 to 883 (with the exception of only 33 oocytes in the experimental group at 50 weeks).

We agree with the assumption made by Brook and colleagues (9) that depletion of the oocyte population caused by any "idiopathic or environmental factor" is prospectively crucial for advancing the risk of maternal age-dependent aneuploidy. According to our model (19) this would include any alteration interfering with the orderly feedback of the hypothalamic-pituitary-gonadal axis. These alterations may originate from postnatal events, as in the aforementioned studies, or even from prenatal irritation which inhibits germ cell proliferation or the escape from atresia.

TRISOMIC SPONTANEOUS HUMAN ABORTIONS AND MATERNAL AGE

Trisomic conceptuses have a high probability of being aborted earlier or later during gestation (8). Data from cytogenetic studies with early spontaneous abortions were used to analyze mean maternal ages for each type of trisomy (for review, see Ref. 19 and 27). The observations of a bimodal maternal-age distribution for some trisomies were explained by maternal age-dependent and age-independent effects which were assumed to be more or less expressed depending on the pronounced or less pronounced bimodality. This argumentation of an age-dependent/independent etiology was retained in considering the data on spontaneous abortions. Trisomies, e.g., 13, 18, and 21, were considered to show a pronounced increase in mean maternal age, while others, e.g., trisomy 16, showed only a weak increase. Then it was suggested that the latter trisomies should be caused by a more or less maternal age-independent mechanism (27). We argued, however, that these data on differences in mean maternal ages between chromosomally normal and trisomic abortions were misapplied when explaining mechanisms of nondisjunction (19; Fig. 3). Recently, a still more comprehensive study on 954 trisomic abortions has been published (28) and data on mean maternal ages for nearly all chromosomes are now available. In Fig. 4 those 16 autosomes which were detected in at least 10 trisomic spontaneous abortions are plotted. Mean maternal age of chromosomally normal, spontaneous abortions (27.0 yr; from the New York study) was delineated at zero. According to our model of a maternal age-dependent increase of trisomy (19) we suggest that a continuum of "gerontological alterations" steadily increases the probability of the occurrence of nondisjunction. Hence, those bivalents will be first affected by nondisjunction carrying, due to inherent individual chromosomal properties, the highest risk of being involved. Such chromosomal properties are, as discussed already, either length/number of chiasmata, nucleolus organizers, or any other property, i.e., constitutive heterochromatin or certain DNA sequences, destining a bivalent to be a "late segregator." (One has to consider, however, that the data may not directly reflect meiotic events, because of the interaction of mutation and selection after fertilization.) According to Fig. 4, chromosomes 4, 6, 8, etc. are considered to be "late segregators," whereas chromosomes 18, 20, and 22 are "non-late segregators," only involved perhaps in a somewhat modified situation at higher ages under worse situations (19; see Fig. 2b).

The pattern of "late segregators" of human chromosomes is, however, only applicable in cases of such trisomies which originate from nondisjunction during (maternal) meiosis 1 (or eventually, meiosis 2), but not from

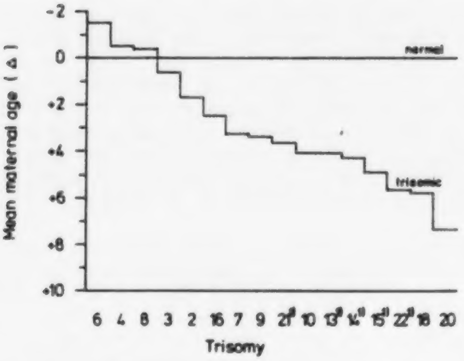

Fig. 4. Sequence of chromosomes according to the difference in mean maternal age (△) of trisomic spontaneous abortions (data from Hassold et al. [28]) in comparison to chromosomally normal abortions (control). Chromosomes with a high △ value are considered "non-late segregators" (e.g., chromosomes 18 and 20); those with a low △ value are considered "late segregators" (e.g., chromosomes 4 and 6), which are at risk to be first involved when the maternal age-dependent causes for nondisjunction steadily increase at younger ages (see also text). Chromosomes carrying a nucleolus organizer are marked 1).

presegregation or chance segregation. The sequence of "late segregators" from human chromosomes (large to small) is in good accordance with the sequence obtained experimentally with the aforementioned Djungarian hamster studies with gonadotrophins and spindle inhibitors, as well as those on the influence of maternal age. Fig. 5 considers just one chromosomal property, length, as a factor for an individual risk for each bivalent to be involved in nondisjunction and, accordingly, the relative length at mitosis and the mean maternal age of trisomic abortions inversely correlate in a significant manner. Chromosomes 16 and 21, for example, seem to have a higher risk for age-dependent nondisjunction than expected from their length only.

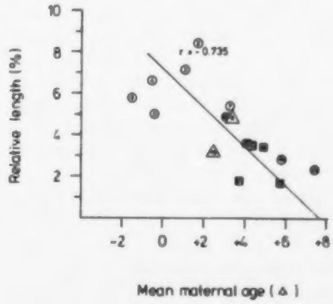

Fig. 5. Correlation of mean maternal age (△) with relative length of each chromosome in mitotic metaphases (from Passarge [45]). Significant (n=16; r=-0.735; m=-0.95) inverse correlation with length of chromosomes (□ = NOR-carriers: chromosomes 13, 14, 15, 21, 22; △ = carriers of prominent constitutive heterochromatin: chromosomes 9 and 16; ○ = other chromosomes: 2-4, 6-8, 10, 18, 20) observed in more than 10 trisomic abortions (28).

Other reasons, like constitutive heterochromatin or the presence of nucleolus organizers on chromosome 21 (see also the discussion on chromosome 5 from Djungarian hamsters), may explain this predisposition.

CONCLUSIONS

The experiments reviewed here* demonstrate that the animal models such as the Djungarian hamster and the NMRI/Han mouse are useful systems for studying basic mechanisms preceding and causing aneuploidy. It was shown that the combined study of the 3 experimental conditions, namely (a) hypergonadotrophic follicular maturation, (b) influence of specific spindle inhibitors, and (c) maternal age, is providing us an insight into processes of nondisjunction and hence also into an orderly chromosome segregation in mammalian meiosis. The events described seem to be of relevance also for the generation of aneuploidy in man as discussed, although only briefly, in the context of trisomic spontaneous abortions. There are now possibilities to investigate differentiation processes causing aneuploidy in mammalian oocytes. Hopefully this understanding may help us to prevent the occurrence of aneuploid conceptions in the future, while at present, we are limited to treating the symptoms caused by aneuploidy.

ACKNOWLEDGEMENTS

Thanks are due to Mrs. M. Heinze-Sprotte for all the editorial work and to Mrs. Gilat for the photographic reproductions. Much of the work reviewed here was part of the Diploma and Doctorate theses of F. Beermann, Edith Hummler, Ingrid Täger, and F. Theuring. These studies have been supported by the Deutsche Forschungsgemeinschaft, the Bundesministerium für Forschung und Technologie (UC/CMT), and Euratom.

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* We intended to review just briefly the recent work done in our laboratory on nondisjunction in germ cells, without discussing it in the context of all the literature published. Comprehensive reviews on spontaneous and induced aneuploidy appeared recently in Ref. 7, 19, and 50.

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DISCUSSION

HOOK: Would anyone comment on the use of mice as opposed to the Djungarian hamster or some other species?

RUSSELL: As I understand Dr. Hansmann, he is suggesting that heteromorphic karyotypes are most likely to nondisjoin. The greater the number of relatively long chromosomes, the greater the amount of asynchronous segregation and the greater the chance of inducing nondisjunction. Has the relation between spread in chromosome lengths and asynchronicity been demonstrated as a general phenomenon? For nondisjunction that is related to recombination, it is the smallest (not the longest) chromosomes that would have the greatest chance of missegregating. Thus, different karyotypes may be suitable for different stages of treatment.

BOOTHROYD: Is any work being done with the tobacco mouse which does have large and small chromosomes as the result of Robertsonian translocations? A comparison there with the standard lab mouse might be quite informative.

RUSSELL: Many Robertsonian translocations from the tobacco mouse, *Mus poschiavinus*, have been introduced into lab strains. Spontaneous nondisjunction frequencies for such translocations can be very high to begin with. Surprisingly, Gropp and Winking have found frequencies several times higher in females than in males. Furthermore, there is a marked age effect on the spontaneous rate in females (but not males) of some Robertsonian-carrying strains.

MECHANISMS AND DETECTION OF CHROMOSOME MALSEGREGATION USING DROSOPHILA AND THE YEAST SACCHAROMYCES CEREVISIAE

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INTRODUCTION

Chromosome aneuploidy in humans may have severe consequences in the individuals in which it occurs or in the following generation (35; Evans, this Volume). Approximately 15% of recognized pregnancies result in spontaneous abortion (78). Approximately half of these result from chromosome abnormalities (13,67). The rate of aneuploidy may actually be much higher than this because early fetal losses may go undetected (12) and certain types of aneuploidy are underrepresented in this sample (5). In fact, the frequency of aneuploids among human sperm is 1.5-5% (47; Martin, this Volume). Among livebirths the frequency of aneuploidy is roughly 0.3% (4). Most of these result from trisomy of one of three autosomes, primarily chromosome 21 (Down syndrome), or from trisomy or monosomy for the sex chromosomes. Although it is generally assumed that meiotic nondisjunction is responsible for the aneuploidy in these spontaneous abortions and birth defects, mitotic aneuploidy may contribute substantially to these incidences. Mason (49) and Vig (77) have pointed out that mitotic nondisjunction in the germline will mimic meiotic nondisjunction, which may serve to explain some otherwise anomalous observations such as the paucity of monosomics among early abortuses and the high frequency of apparent meiosis 2 errors.

Mitotic chromosome malsegregation may play a role in the etiology of neoplastic transformation. Aneuploidy for specific chromosomes has been repeatedly reported for particular neoplasms, and correlated with the localization of oncogenes of other genes associated with particular malignancies (see Evans, this Volume). At least in one case, treatment of cells at mitosis with an aneuploidy-inducing agent results in the parallel induction of aneuploidy and cell transformation (76). The degree to which human abnormalities are due to exposure to environmental agents is unknown. Although no agents have been shown to induce aneuploidy in humans, several have been identified in other species. It is entirely possible that one or more of these agents may have similar effects in humans.

The term aneuploidy has been used to describe two different kinds of phenomena. The first of these, partial or segmental aneuploidy, corresponds to a disproportionate representation of segments of chromosomes while chromosomal aneuploidy, which is discussed here as aneuploidy, refers to a disproportionate representation of whole chromosomes. The latter is considered to arise primarily from chromosome malsegregation, although other mechanisms such as overreplication of single chromosomes cannot be ruled out.

PROCESSES IMPORTANT FOR CHROMOSOME SEGREGATION

In spite of the fact that considerable effort has been expended toward understanding mitotic and meiotic chromosome segregation, there are large gaps in our knowledge of this process. Three basic approaches have been taken to elucidate the mechanisms of chromosome segregation. The first was a cytological description of the chromosomes themselves starting in the late 19th century followed by a description of mitosis and meiosis. This cytological analysis was aided greatly by the introduction of the electron microscope which allowed a detailed description of several components of the segregational apparatus, e.g., microtubules and the synaptonemal complex. The second approach, a genetic dissection, which began with the discovery that chromosomes are the genetic material (9), was driven by the use of meiotic mutants (1); mutants have been identified which can affect mitotic (29) and/or meiotic segregation. To a large degree, genetic dissection is based on the previous cytological description. The biochemistry of meiosis has been described elsewhere in some detail (73). This approach, involving the relationship of biochemical events to chromosomal segregation, will probably become very informative in the near future as it is coupled with genetic analyses (Resnick, in prep.). A fourth approach that has not been used extensively involves the use of chemical probes to elucidate mitotic or meiotic chromosome segregation mechanisms; coincident with this approach are the identification and characterization of chemicals that cause aneuploidy. A number of spindle poisons have been identified and used to disrupt microtubules, but a more thorough toxicological analysis of meiosis or mitosis has not been attempted.

Chemicals that disrupt timing or structure of the segregational apparatus might be expected to produce chromosome aneuploidy as one endpoint. Unlike mutagens whose primary target is DNA, chemicals that induce aneuploidy should include those with divergent activities and a large number of targets. These targets could be defined in a broad sense by what we already know about the processes of mitosis and meiosis which can be broken down further into their physical components. To a large extent any discussion of these components and how they might be affected by exogenous chemicals is necessarily speculative.

One of the earliest steps in the mitotic/meiotic process is chromosome packaging or condensation. Not only is condensation necessary for the sorting out of chromosomes at anaphase, but a distinct configuration may be necessary for proper centromere-spindle fiber associations to occur. Specialized chromatin configurations associated with the synaptonemal complex and the telomeres may also be important. All of the structural components of chromatin, including RNA, DNA, and protein, are likely to play a role.

Chromosome pairing can take many forms. In mitosis sister chromatids are held together at their centromeres. In addition to a sister-chromatid association, the synapsis of homologous chromosomes at meiosis is also important for proper separation at anaphase 1. Not much is known about this process, although tubulin has been implicated as a component (23,24,70). Telomeric associations with the nuclear membrane may also play an important role at this stage. Genetic evidence in Drosophila suggests that a site near the end of the X chromosome may be important for recombination (33,75).

Chromosome segregation is also dependent on several DNA metabolic processes, including replication, recombination, and DNA repair, all of which could be the targets of chemicals that induce aneuploidy. For example, in many species, premeiotic S-phase is longer than the S-phase of mitotically dividing cells; heat shocks administered during premeiotic S-phase can drastically affect the frequency of meiotic recombination (2,32), suggesting that a precondition for exchange may be established during S-phase. Meiotic recombination, of course, has long been known to be required for proper segregation. Bridges (9), for example, showed that the chromosomes that nondisjoin are primarily nonrecombinant. Since the synaptonemal complex and structures known as recombination nodules play a role in recombination (15,16), they would be expected to be important for chromosome segregation. DNA repair may also play an important role for proper segregation by protecting the DNA from damage that might interfere with either recombination, as shown for yeast (64), or directly with segregation; for example, DNA damage occurring at the centromere may directly affect segregation (Resnick, Westmoreland, and Bloom, unpubl. observ.). Variations in nucleotide pools can affect segregation (42); these pools can be influenced directly or by energy metabolic processes. Structural chromatin-associated proteins might also be expected to play a central role.

A secondary pairing process has been described in Drosophila that acts independently of synapsis to allow segregation of nonrecombinant chromosomes (31). This process, termed distributive pairing, has not been described in other organisms, but must still be considered as a possible target for chemicals that induce aneuploidy.

Several of the components involved in the actual segregation of chromosomes have been characterized. At metaphase the spindle fibers are attached at one end to a chromosomal centromere, and at the other they end in the centrosome which surrounds the centriole or spindle pole body. Support is provided by other microtubules that span the region between the centrosomes and by asters that surround the centrosome. Some of the components may serve multiple functions. In meiosis, for example, the centromere must: (a) attach to the microtubule, (b) orient homologous chromosomes at metaphase 1, (c) allow homologs to separate at anaphase 1, while holding sister chromatids together, reorienting at metaphase 2, and allowing sisters to separate at anaphase 2. Many of these functions can be separated genetically. Meiotic mutants have been identified in Drosophila that, for example, are defective in holding sister chromatids together during meiosis 1 and thus exhibit high frequencies of nondisjunction at the second and possibly even the first division of meiosis (19,42).

Several chemical probes have been identified which appear to affect specifically the nonDNA portion of the segregational apparatus. Chemically induced aneuploidy may also occur indirectly. To the extent that any of the steps in segregation are calcium-dependent, they may be regulated by calmodulin. Calmodulin levels have been observed to increase at the beginning of S-phase in rat liver cells (7,17). Calmodulin has also been implicated in the control of tubulin stability (46). Therefore, chemicals that affect calmodulin levels or activity may contribute to the induction of aneuploidy. Chemicals that affect hormone levels may also contribute to aneuploidy. Tsutsui et al. (76) have found that synchronized cells show a peak of sensitivity to diethylstilbestrol at mitosis for the induction of both aneuploidy and transformation.

The observation with diethylstilbestrol suggests that nonmutagenic carcinogens may act indirectly at the chromosomal level by inducing aneuploidy rather than specific changes in DNA sequences. It is reasonable to suppose, therefore, that in identifying and characterizing agents which induce aneuploidy there is an opportunity to gain insight into some of the possible mechanisms of carcinogenesis as well as understanding the mechanisms of chromosome segregation.

GENETIC STUDIES IN DROSOPHILA AND YEAST

There are features common to both yeast and Drosophila which make these organisms particularly well-suited to studies of chromosome stability and the impact of chemicals. They are genetically well-characterized, mutants and agents affecting chromosome stability can be examined for their effects in both mitotic and meiotic cells, both chromosome gain and loss can be measured, other genetic effects including mutation and recombination can be evaluated, and it is potentially possible to use mutants to distinguish between targets for chemical action. As discussed below, the latter point is best illustrated for the case of suspected DNA-damaging agents.

There are at least two genetically and biochemically identifiable systems for repairing DNA damage based on responses to UV and ionizing radiation (for reviews of yeast systems see Ref. 27 and 34). In addition, several genes have been identified by sensitivity of mutants to other mutagenic agents (6,41,60,71). If DNA lesions are a major source of aneuploidy, mutants lacking repair capabilities might be expected to exhibit high levels of aneuploidy in response to mutagen treatment, but not in response to agents which damage other targets. Although there are indications that this is the case, experiments to clearly demonstrate this concept remain to be done. An excision-defective mutant of yeast appears to be more sensitive to UV-induced aneuploidy (unpubl. observ.); however, because UV induces recombination which could mimic malsegregation the effect of UV is not clear. Similarly, an excision-defective mutant in Drosophila appears to increase the frequency of mutagen-induced chromosome loss (79), although this has not been confirmed using a centromere-linked marker.

The mechanism by which DNA damage could cause aneuploidy can only be speculated at this point. Likely possibilities include (a) effects on recombination, (b) damage at a site which is physically involved in segregation, e.g., the centromere, and (c) heterologous interchanges inducing regular segregation of the interchange chromosomes and random assortment of the respective homologs (59). As mentioned earlier, recombination is important for normal disjunction during meiosis, and any interruption of the process leads to enhanced aneuploidy. Mutants have been identified in both Drosophila and yeast which result in both loss of meiotic recombination and an increase in chromosome malsegregation; these include mei-9 and c(3)G of Drosophila and rad50 and spo11 in yeast. In yeast this has been demonstrated using the spo13 mutation in combination with rad50 or spo11 (44,45). A mutation in the spo13 gene (39) results in a bypass of meiosis I reductional division thereby eliminating the requirement for recombination; the only meiotic division is similar to that in mitotic cells. The spores which arise after meiosis in rad50 or spo11 strains are inviable, while rad50 spo13 and spo11 spo13 mutants give rise to diploid spores that do not exhibit recombination. These and other mutations lacking meiotic recombination may be in genes controlling any of several steps in meiotic chromosome metabolism from initiation of chromosome pairing to recombination (1). For example, c(3)G mutants in Drosophila and rad50 mutants of yeast eliminate the synaptonemal complex; the mutations could affect the synthesis of this structure or they could affect earlier steps in a pathway leading to recombination.

In Drosophila an increase in meiotic recombination may also lead to aneuploidy. Diplo-X ova resulting from spontaneous nondisjunction have been examined for the presence of recombinant X chromosomes (52). This examination showed that these ova developed from meiocytes that either had no X-chromosome recombination or had undergone multiple exchanges. In contrast, ova in which the X chromosomes segregate normally develop from meiocytes that have had primarily a single exchange on the X chromosome. In other words, spontaneously occurring nondisjunction can result from one of two conditions: either the chromosomes that nondisjoin have not been involved in exchange and do not orient properly at metaphase or they have recombined multiple times and are thus unable to separate. Merriam and Frost (52) also concluded that spontaneous mitotic and meiosis 2 nondisjunction are very low compared with meiosis 1 nondisjunction in Drosophila. Mitotic nondisjunction has also been shown to be lower than meiotic nondisjunction in yeast (66).

The role of recombination in chromosome malsegregation in mitotic cells is less clear, although 2 lines of evidence from yeast suggest its importance. Campbell and Fogel (11) showed that when selection was made for recombination near the centromere of chromosome 3 there was frequent associated aneuploidy. There was no such association with recombination at genes far removed from the centromere. Additional evidence comes from observations with rad52 mutants of yeast. These mutants exhibit reduced levels of spontaneous mitotic and meiotic recombination compared with wild-type controls, and do not give rise to X-ray-induced recombination (28,61, 63). The few spontaneous recombinants that are recovered are associated with broken chromosomes. The rad52 mutants are also defective in chromosomal double-strand break repair which has been proposed to occur through recombination (62), and they yield high levels of spontaneous and X-ray-induced mitotic chromosome loss (53). One interpretation of these results is that unresolved recombination intermediates can lead to aneuploidy.

While many of the known mutants disrupting chromosome segregation interfere with some aspect of DNA metabolism, particularly in meiosis, there are several examples of mutants that affect other cellular structures. The nod mutation in Drosophila increases nondisjunction by disrupting distributive disjunction (14). Another mutant in Drosophila, which has a defect in a testis-specific β-tubulin, increases the frequency of meiotic nondisjunction (Kemphues and Kaufman, pers. comm.). The effects of this mutation and others at the same locus can be mimicked by the use of colchicine (38). Similarly, a tubulin mutant in yeast results in enhanced aneuploidy (56). Another interesting class of mutants is that which affects membrane synthesis; in yeast a high level of aneuploidy in these mutants could be related to chromosome-membrane interactions. Other mutants are known which specifically interfere with the ability of centromeres to remain intact during meiosis in Drosophila (21,48). These mutants may disrupt segregation at the first and/or second meiotic division, depending on the time at which centromeres dissociate (30), and may even disrupt meiotic recombination (48). Such meiosis-specific centromere mutations are to be expected because the first division of meiosis is the only cell division in which sister chromatids do not segregate from each other.

Telomeres are other chromosomal organelles that may be important for chromosomal segregation. Telomeres and their associated DNA sequences are complex structures that can be subdivided into discrete subregions (18). It is now possible to delete telomere-associated sequences and replace them with new, still undefined sequences (50). The effects of such telomere deletions on meiotic recombination and chromosome segregation are currently under investigation.

TOXICOLOGY

Because of the lack of adequate assay systems for detecting aneuploidy (22), the first step in the search for chemicals that induce aneuploidy, which can be used to investigate mechanisms of chromosome malsegregation, is the development of systems designed to screen large numbers of chemicals for their aneuploidy-inducing ability. Several assays for chemically induced aneuploidy have been developed recently in Drosophila (reviewed in Ref. 25 and 81). Three Drosophila assays are currently being considered for use in a screen for chemicals that induce aneuploidy (80). All three assays share a number of desirable attributes. In each case, females are treated with the test chemical because meiosis in Drosophila females closely resembles meiosis in other species. Larvae are treated, so both oogonia and oocytes may be exposed to the test chemical and its metabolites. These assays measure both the gain and loss of sex chromosomes and allow at least one regular progeny class to survive, so aneuploid frequencies can be calculated. The females used in the three assays differ in the constitution of their sex chromosomes. In two of the assays recombination between homologs is reduced through the use of heterozygous inversions or an attached-X/Y homologous pair. Thus, the segregation of the sex chromosomes in these females should be more sensitive to the level of autosomal recombination through the action of the distributive pairing system. The third assay can distinguish equational nondisjunction from reductional nondisjunction and may, thus, allow one to determine the division at which malsegregation occurs.

Assays have been developed in yeast (see Ref. 65) to test for aneuploidy induced during meiosis based on chromosome gain and during mitosis based on chromosome loss. Chromosome gain may also be detected by gene dosage effects (66). Although the purpose of these assays is to screen for chemically induced aneuploidy, the end products themselves may not be aneuploid. The cells of a yeast colony may be euploid (74,82), although they express specific markers suggestive of an earlier aneuploid condition. Similarly, gametic aneuploidy in Drosophila is inferred from genetic markers expressed in the adult progeny.

Several chemicals that induce aneuploidy have been found in yeast and Drosophila which do not induce mutation or recombination. These would appear to exert their effects through targets other than DNA. It is important to note that standard "mutation" assays (such as the Ames assay) would not pick up these important mutagenic agents, which include solvents such as DMSO and acetone, and tubulin-interacting agents such as nocodazole, benomyl, and colchicine. The effect of aprotic polar solvents in mitotic cells appears to be due to modification of tubulin interactions during either assembly or disassembly (83). Several aprotic polar solvents have now been identified which specifically cause chromosomal malsegregation, including acetone, acetonitrile, and ethyl acetate (82).

DNA damage has been implicated as a source of aneuploidy by the observation that known mutagens and clastogens are capable of inducing aneuploidy. For example, 8 of 11 chemicals that have been shown to induce aneuploidy also induce chromosome aberrations and SC exchanges (26). Similarly in Drosophila and yeast, several of the chemicals considered to induce aneuploidy are also mutagens and/or recombinogens (65,81). They have been shown to cause both chromosome gain and loss. Ethyl methanesulfonate is an efficient inducer of recombination in yeast. As shown by Campbell (10), it also induces aneuploidy. Similar to the case for spontaneous aneuploidy, ethyl methanesulfonate-induced recombination near the centromere is frequently associated with aneuploidy.

Specific effects of DNA damage on meiotic recombination and chromosome segregation have come from experiments with UV-irradiated yeast. Pyrimidine dimers induced in DNA at the beginning of meiosis reduced meiotic recombination as measured in interrupted meiosis experiments (see Ref. 64). If meiotic development were allowed to go to completion so that the spores (i.e., gametes) were formed, the spores were inviable. These results were interpreted as being due to a suppression of meiotic recombination by DNA damage which then leads to chromosomal malsegregation. The damage could have affected pairing via the synaptonemal complex or, alternatively, directly inhibited reciprocal exchange events through interference with the exchange process. In Drosophila, X-rays increase the frequency of centromere proximal meiotic recombination, decrease the frequency of distal recombination, and increase the frequency of nondisjunction (51,54). However, the relationship between effects on recombination and segregation are not clear. It has been proposed that X-rays interfere with chromosome pairing in distal regions and that the associated decline in recombination is responsible for the increase in nondisjunction (19,68). Alternatively, Muller (55) proposed that an increase in recombination in the centromere region was responsible for the nondisjunction.

The centromere is currently under investigation as a site of damage important for induced aneuploidy in yeast (Resnick and Bloom, in prep.). Centromeres of yeast consist of a region less than 250 bp which is essential for proper chromosome segregation; associated with this is a protein(s) that appears to be different from that involved in nucleosomes (3). The sensitivity of the region has been examined using UV as a specific inducer of DNA damage. Hot spots for pyrimidine dimers have been demonstrated within, as well as near, the 250-bp region; the hot spots appear to correlate with AT-rich stretches of DNA. Preliminary results indicate that in vivo centromere conformation reduces the effect of UV because pure centromeric DNA irradiated in vitro is more sensitive than chromosomal DNA irradiated in vivo. Based on these measurements it appears that at biological doses (i.e., >10% survival) there is an average of approximately 0.1 to 0.2 dimers per essential centromere region. The effect of centromeric DNA damage on chromosome stability is currently under investigation.

AGING AND ANEUPLOIDY: IMPLICATIONS FROM YEAST AND DROSOPHILA

The genetic approaches with yeast and Drosophila, which have allowed DNA and nonDNA targets to be distinguished, strongly implicate DNA-related events in the production of aneuploid cells. As noted above, these events could arise from effects of recombination, damage to specific DNA targets, and deficiencies in DNA repair. It can also be proposed that altered DNA synthesis, such as errors in replication of essential regions or incomplete replication of structures such as telomeres or centromeres, would also affect proper chromosome disjunction.

While an aging component in aneuploidy has been established (see other chapters in this Volume and review in Ref. 4), the underlying causes are not understood. As summarized above, DNA-related events and processes can contribute significantly to chromosome malsegregation. To the extent that aging affects DNA metabolism and the accumulation of DNA damage, changes would be expected in chromosome distribution. For example, oocytes arrested in the dictyate stage may have reduced repair capabilities as compared to other germline cells or they may be more subject to perturbations which would be revealed in terms of aneuploidy. It is clear from various studies on gametogenesis that enzymes which may play a part in recombination and/or repair vary with the meiotic cycle (20,36,58) as well as repair capabilities (40,72). Possibly, the dictyate stage has less or a different combination of these enzymes so that reduced capabilities for repair may exist. Evidence already exists for reduced repair capabilities in somatic cells from older as compared to young individuals (37 [and refs. therein], 57).

The mechanisms by which altered repair and recombination capacity could affect chromosome segregation can be inferred from the various mutants and studies with yeast and Drosophila described earlier. An intriguing possibility exists that involves additional recombinational interactions at the dictyate stage. Since chromosomes are closely associated over several years in human oocytes, it is conceivable that either accumulated damage, recombinational repair processes, or spontaneous events might lead to additional chromosomal interactions over and above that which take place in meiotic prophase. The additional or even defective recombinational events might lead to aneuploidy as has been found for the case of mitotic aneuploidy induced by ethyl methanesulfonate in yeast (10) or in mutants defective in the ability to complete recombination (53).

Accumulation of DNA damage, particularly if there is reduced repair capability, could strongly influence chromosome segregation, especially if it occurred in critical targets. Not only could single-strand damage become significant, but there is the possibility of double-strand events. For example, sufficient damage might accumulate on both strands such that repair processes could lead to double-strand breaks. Repair related double-strand breaks have been demonstrated for UV-damage in human cells (8).

One type of naturally occurring lesion in DNA that might lead to secondary events, which in turn could influence chromosome segregation, is the apurinic gap in DNA. At 37°C, there is a natural depurination rate of approximately 5,000 to 10,000 events per cell per generation (43); the rate of loss of pyrimidines is about 10 times less. Based on this rate of loss, the expected frequency of events over 30 years is approximately 3 per 1,000 bases. Within a region of 1,000 bp, there would be an expectation of 0.2 double events on opposite strands that are within 10 bp of each other (69). While growing cells are extremely efficient at repairing apurinic gaps through a process involving an endonuclease followed by a small region of excision repair, it is conceivable that such repair is reduced in all or parts of dictyate chromosomes so that gaps might accumulate and possibly even double-strand gaps in close opposition might occur. In addition, there may be the possibility of misrepair.

It can therefore be seen that chromosomal DNA and metabolism may be important sites and processes which are affected by the aging process. Based on results from lower organisms, mechanisms can be proposed by which aging influences chromosome segregation. The insight gained should stimulate investigations with mammalian systems.

CONCLUSIONS

Yeast and Drosophila can be considered as ideal for examining and interrelating several issues related to aneuploidy. They allow for combined studies of genetic and molecular controls of chromosome segregation, the nature and effects of chemicals that induce aneuploidy, the relationship between mitotic and meiotic events, and the importance of other genetic processes. It is important that, in terms of sensitivity, the levels of detection for yeast and Drosophila may render measurements in these systems more meaningful than cytological observations which require relatively high doses of agents to detect effects. Typical spontaneous rates of chromosome malsegregation in these organisms are in the range of 10^{-3} to 10^{-6} per division, and induced frequencies of 10^{-3} to 10^{-4} are readily detectable genetically. Because concerns about aneuploidy may relate to genetic consequences in viable offspring, it is important to measure effects at low, biologically meaningful doses, rather than at high doses which may be necessary for the cytological detection of changes in chromosome number.

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SPECIAL COMMITTEE REPORT, PART I: AN EVALUATION OF CURRENT TESTING APPROACHES FOR THE DETECTION OF CHEMICALLY INDUCED ANEUPLOIDY

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INTRODUCTION

Evaluation of scientific data to determine the likelihood that a particular chemical may pose a health risk is a major function of the U.S. Environmental Protection Agency (EPA). In certain legislative mandates (e.g., Toxic Substances Control Act), mutagenicity is identified as a toxicological endpoint for which testing standards must be developed. Thus, the EPA must make decisions on testing methods for mutagenicity. Most genetic toxicology tests identify chemicals that cause gene mutations, chromosome breakage and rearrangement, or other forms of direct DNA damage. Because DNA is not necessarily the target for the induction of numerical chromosome mutations, induction of aneuploidy may not correlate with the induction of other types of genetic alterations. Tests designed specifically to detect aneuploidy should be included in a screening or testing program to better characterize the potential genetic (or carcinogenic) hazard of a chemical.

ANEUPLOIDY DATA REVIEW COMMITTEE

Seven years ago, the Gene-Tox Program, sponsored by the EPA, initiated an evaluation of the status of bioassays in genetic toxicology, including aneuploidy tests on Drosophila and fungal systems (14); aneuploidy tests using plants or mammalian systems were not reviewed. In 1984, an expert

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* Operated by Martin Marietta Energy Systems, Inc. under contract No. DE-AC05-80R 21400 with the U.S. Department of Energy.

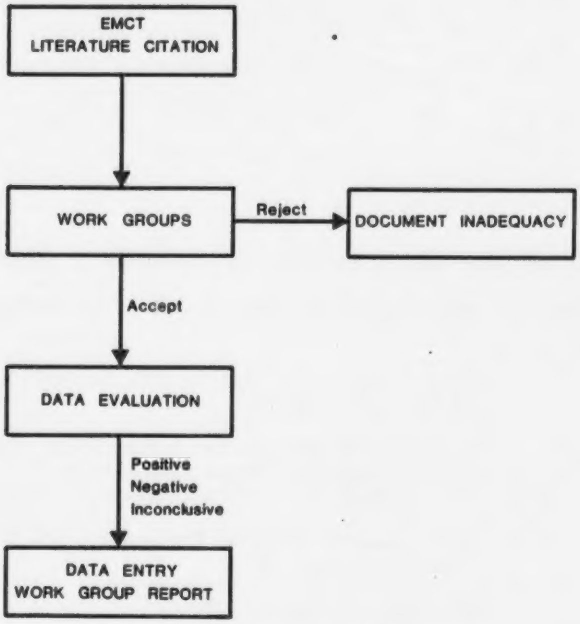

Fig. 1. The Aneuploidy Data Review Committee evaluation process.

committee, the Aneuploidy Data Review Committee,* was established by the EPA to identify the testing approaches currently available for the detection of chemically induced aneuploidy and to appraise this methodology (5). This Committee is organized into 9 working groups (Tab. 1). An evaluation of the available test systems was accomplished through a critical analysis of the published literature. The Aneuploidy file produced by the Environmental Mutagen, Carcinogen, and Teratogen Information Program (EMCT) at the Oak Ridge National Laboratory was the principal resource for identifying papers to be reviewed (9). This bibliographic database covers the literature on aneuploidy from 1970 to the present. Information not published in the open literature was included if it was available through EMCT or the National Technical Information Service (Springfield, Virginia).

The evaluation process is depicted in Fig. 1. Initially, the working groups screened the papers provided, and rejected those with obvious inadequacies (e.g., lack of relevant data or insufficient data to make an independent evaluation of the results, lack of control data, or improper protocols). The individual working groups imposed additional criteria for rejection that were specific for their test systems. Because the review was directed toward individual chemicals, papers on nonchemical agents, combinations of agents, or complex mixtures were excluded. More than 1,000 papers were provided to the Committee: over half of these were eliminated during the first cut of the literature. The reasons for rejection of papers have been documented at the EMCT.

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* This Committee was directed by the Reproductive Effects Assessment Group and the Health Effects Research Laboratory within the Office of Research and Development, U.S. Environmental Protection Agency.

Tab. 1. The Aneuploidy Data Review Committee.

Organization	Committee member(s)	Affiliation
Coordinators	Vicki L. Dellarco Jan C. Liang Michael D. Waters	U.S. Environmental Protection Agency University of Texas U.S. Environmental Protection Agency
EMCT	Kathleen H. Mavournin	Oak Ridge National Laboratory
Working groups		
Saccharomyces	Vernon W. Mayer Michael A. Resnick F.K. Zimmermann	Food and Drug Administration Natl. Inst. of Environ. Health Sciences Technische Hochschule, West Germany
Aspergillus	Etta Käfer Andreas Kappas Barry R. Scott	McGill University, Canada Nuclear Research Center, Greece Phoenix Lone Oak Labs, Texas
Neurospora	Herman E. Brockman David M. DeMarini Frederick J. de Serres A.J.F. Griffiths	Illinois State University U.S. Environmental Protection Agency Natl. Inst. of Environ. Health Sciences University of British Columbia, Canada
Higher plants	Milton J. Constantin Shabeg Sandhu Baldev K. Vig	University of Tennessee U.S. Environmental Protection Agency University of Nevada
Drosophila	James M. Mason Chris Osgood Stanley Zimmering	Natl. Inst. of Environ. Health Sciences University of Kentucky Brown University, Rhode Island
Mammalian cells in culture	James L. Ivett Sheila M. Galloway	Litton Bionetics, Inc., Maryland Merck, Sharpe, Dohme, Pennsylvania
Mammalian somatic cells in vivo	Michael C. Cimino Jan C. Liang Raymond R. Tice	U.S. Environmental Protection Agency University of Texas Brookhaven National Laboratory
Mammalian male germ cells in vivo	James W. Allen Anthony V. Carrano Jan C. Liang R. Julian Preston	U.S. Environmental Protection Agency Lawrence Livermore National Laboratory University of Texas Oak Ridge National Laboratory
Mammalian female germ cells in vivo	K.S. Lavappa John B. Mailhes R. Julian Preston	Food and Drug Administration Louisiana State University Oak Ridge National Laboratory

The remaining papers were evaluated further by the working groups, and data were classified as positive, negative, or inconclusive. The criteria used to classify results were stringent for determining malsegregation. The reports of individual working groups provide descriptions of test approaches, general principles to be followed in designing test protocols, criteria to evaluate data, and recommendations for future studies in the area of aneuploidy test development. They will be published together in a special issue of Mutation Research. This chapter provides an overview of the findings and conclusions of the Aneuploidy Data Review Committee.

TESTING APPROACHES

Several methods have been developed to assay for the induction of mitotic or meiotic aneuploidy. The nonmammalian systems usually are genetically based. In Aspergillus, various types of mitotic aneuploids are detected (8), whereas in Neurospora, chromosome gain is measured following meiosis (7). In Saccharomyces, approaches have been devised to detect either chromosome loss or gain during mitosis or meiosis (11); however, more chemicals have been evaluated for mitotic chromosome loss. Testing schemes in Drosophila measure meiotic loss or gain in females or males (15). Certain nonmammalian systems (e.g., Sordaria, Melanopius) were not evaluated because relatively little data has been published. These methods should be further investigated. Plant systems and mammalian systems usually employ cytogenetic analysis (1,3,6,10,13). Some genetic methods for mammals have been devised (Ref. 12 and Russell, this Volume).

Aneuploidy tests are at different stages of test development, but none have been used enough to meet commonly accepted standards of test validation (i.e., reproducibility within a given laboratory, agreement of results among different laboratories, acceptable level of concordance with other test systems, confirmed ability to detect the endpoint in question). Because test development and data generation have progressed slowly in this area, the analysis of test results could not be based on standardized protocols. Moreover, the Aneuploidy Data Review Committee considered it premature to recommend standard protocols when factors such as optimum sampling times and appropriate positive controls were undetermined.

CURRENT STATUS OF THE DATABASE ON THE CHEMICAL INDUCTION OF ANEUPLOIDY

The current database is still too incomplete to support comparative analysis among different test systems. About 140 chemicals were evaluated as negative or positive in at least one of the 9 test systems (Fig. 2). The number decreases to about 35 chemicals with results in 2 different test systems, and to about 20 chemicals evaluated as negative or positive in 3 assays. Cyclophosphamide and benomyl were the only chemicals that had results evaluated in 4 or more test systems (see Tab. 2). Cyclophosphamide was positive in Saccharomyces, Aspergillus, and in both male and female germ cells in whole mammals. Cyclophosphamide is a DNA-damaging agent, and in Aspergillus it produces malsegregation as a secondary effect to chromosome breakage. Benomyl, a carbamate pesticide, was evaluated as positive in Aspergillus, Saccharomyces, and mammalian cells in culture. Cyclophosphamide and benomyl were found to be negative in Neurospora.

Figure 3 illustrates the numbers of chemicals evaluated as positive, negative, or inconclusive. Very few chemicals could be classified as positive; these are listed by test system in Tab. 2. It should be emphasized that many of these chemicals only had positive results from a single study and these should be verified. Many of these compounds induce other genetic alterations in addition to aneuploidy, notably azathioprine, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), ethylene dichloride, methyl nitronitrosoguanidine (MNNG), and cyclophosphamide (4). Several chemicals listed in Tab. 2 have been shown to be spindle disruptors (e.g., Colcemid, colchicine, vinblastine, griseofulvin, diazepam, chloral hydrate, diethylstilbestrol (DES), methylbenzimidazole carbamate (MBC), p-fluorophenylalanine) (see Liang and Brinkley, this Volume).

Some chemicals were evaluated as negative (Fig. 3). These negative evaluations must be qualified because most are based on single studies, or one germ cell stage, or less than toxic test doses. None of the compounds

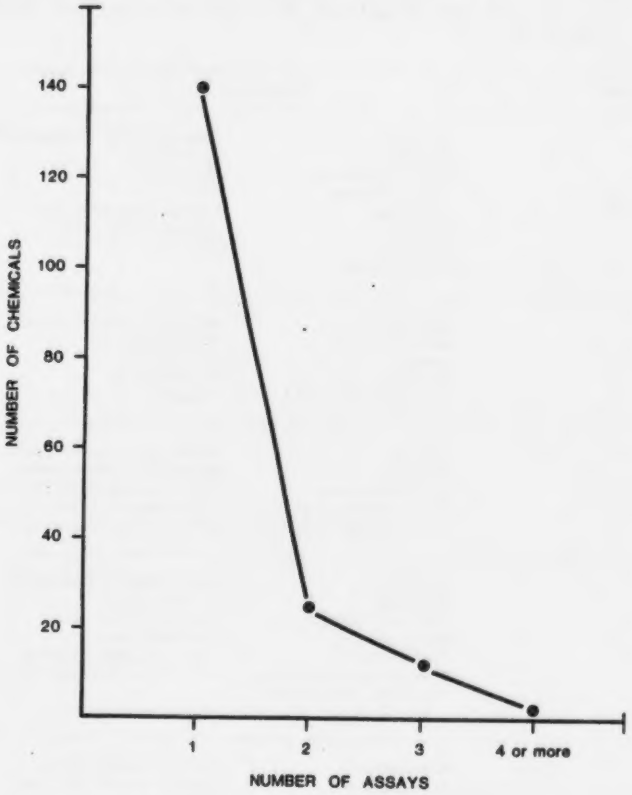

Fig. 2. The number of chemicals that were evaluated either as negative or positive in the 9 test systems, i.e., Aspergillus, Neurospora, Saccharomyces, Drosophila, plants, mammalian cells in culture, whole mammal somatic cells, and mammalian female and male germ cells.

assessed as negative in fungal systems or cultured cells was tested in the presence of an exogenous source of metabolic activation. It is not known whether biotransformation is required for aneuploidy induction by certain chemicals. There were many negatives in Neurospora; some are probably false negatives (e.g., cyclophosphamide and benomyl) because they were positive in several other systems.

The number of inconclusive evaluations is a matter for concern. The private sector, as well as the EPA and other parts of government, relies heavily on the published literature to determine the toxicological potential of certain chemicals, and it is clear that a great deal of published data on the chemical induction of aneuploidy is inadequate or inconclusive. Many of the deficiencies in the literature are very basic and apply across different test systems and include factors such as protocol uncertainties, insufficient documentation of results or protocol, insufficient sample sizes, or lack of control data. In Saccharomyces, most of the inconclusives result from failure to rule out other genetic alterations that can mimic the aneuploid phenotype; because this test system is genetically based, it is important to confirm the aneuploid phenotype by testing for

Tab. 2. Summary of positive chemicals for aneuploidy. These data were derived for the Aneuploidy Data Review Committee Reports (1,3,6, 7,8,10,13,15).

Test system	Chemicals	
Aspergillus*	Amphotericin B Benomyl Chloral hydrate Chloropropham Econazole Ethanol Fenarimol Griseofulvin	Iodochlorohydroxyquinoline Miconazole Natamycin MNNG p-Fluorophenylalanine Sodium deoxycholate Thiram
Neurospora	Amethopterin m-Aminophenol Bromacil Caffeine 2,4-Diaminoanisole	p-Fluorophenylalanine Sulfacetamide Trifluralin Trimethoprim Atrazine
Saccharomyces	Acetone Benomyl Cyclophosphamide Ethyl methanesulfonate p-Fluorophenylalanine	Methyl methanesulfonate Nocodazole (onodazole) 12-O-Tetradecanyl-phorobol-13-acetate (TPA) Methyl-2-benzimidazol carbamate
Plants	Aspirin Atrazine Chloramphenicol Colchicine Cycloheximide Diethyl pyrocarbonate 2,4-dichlorophenoxyacetic acid	Ethyl methanesulfonate Griseofulvin Sodium azide Tetracycline 2,4,5-Trichlorophenol 2,4,5-Trichlorophenoxyacetic acid
Drosophila	Acenaphthene Actinomycin-D Azathioprine Bleomycin Caffeine Clophen A50 Colcemid Halothane 1,2-Dichloroethane	5-Fluorodeoxyuridine Methyl mercury hydroxide MNNG pp'DDT Proflavine Satratoxin-H Sodium cyclamate Vinblastin
Mammalian cells in culture	Benomyl Benzo(a)pyrene Carbaryl Colcemid (colchicine) Diazepam Diethylstilbestrol	Dimethylbenzanthracene Griseofulvin N-Methyl-N-nitronitrosoguanidine TPA Vinblastin
Mammalian somatic cells in vivo	Pyrimethamine	
Mammalian male germ cells in vivo	Cyclophosphamide	Chloral hydrate
Mammalian female germ cells in vivo	Cadmium chloride Colchicine Cyclophosphamide Methotrexate	Methylmercuric chloride Norethindrone acetate Pyrimethamine Triaziquone

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* Compounds that induce malsegregation as a secondary effect are not included in this list (8).

loss of other markers, for aneuploidy of additional chromosomes, or for aberrant tetrad ratios after meiosis. This potential problem applies to genetically devised test schemes in general.

For mammalian somatic cells in vivo, about 95% of the results were classified as inconclusive, primarily because the number of animals or cells per treatment group was not specified. This information is essential to ascertain animal-to-animal variability. A problem, encountered in the literature on all mammalian systems, is the lumping of hyperploidy and hypoploidy data into a single category. Sometimes polyploidy and aneuploidy data were combined. Hypoploidy alone is usually insufficient evidence for aneuploidy induction because of the contribution of artifacts from slide preparation, or from the induction of membrane fragility by the test compound. If hyperploid cells were not observed or reported, the data were considered inconclusive.

In mammalian male germ cells, the major reason for the high number of inconclusives was questionable or uncertain measurements of aneuploidy (e.g., univalency, fluorescent bodies, translocations). At present, there is insufficient evidence to substantiate the relationship of these endpoints to the induction of aneuploidy. Direct chromosome counts were considered by the working groups to be the only acceptable measure of aneuploidy.

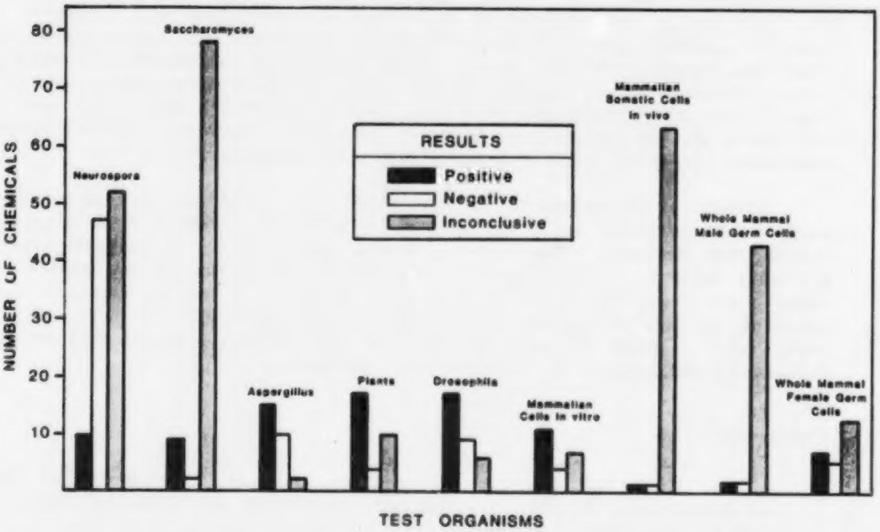

Fig. 3. The number of chemicals evaluated as positive, negative, or inconclusive by the 9 Aneuploidy Data Review Committee working groups.

GENERAL PRINCIPLES TO BE FOLLOWED IN PROTOCOL DEVELOPMENT

The inadequacies of the current database limit its usefulness, but they do provide guidance for protocol development. It is important to test several doses so that the active concentration is not missed, especially if there is a narrow window of activity, and to confirm a positive response by demonstrating a dose-related effect. Some level of toxicity should be achieved for a negative test, to insure that the system has been challenged adequately. Sample sizes will be determined by several factors: nature of the test system (e.g., baseline frequency of aneuploidy), the choice of the statistical analysis, and the desired level of significance. Replication kinetics and sampling times are also critical parameters; treated cells should be examined for aneuploidy after sufficient time for one intervening round of cell division but before the death or contraselection of aneuploid products. The interval between treatment and observation is important in assessing different germ cell stages. Negative and positive controls are essential for any valid investigation; reliable positive control compounds have yet to be determined for most aneuploidy assays. Physical conditions (e.g., pH or temperature) can influence the results and must be controlled (6). Most of all, reliable indicators of aneuploidy should be used to eliminate irrelevant endpoints or genetic alterations that mimic the aneuploid phenotype. The data and protocol should be reported in sufficient detail to permit thorough analysis and independent evaluation of the results.

FUTURE TEST DEVELOPMENT

Most of the test systems reviewed by the Aneuploidy Data Review Committee are in need of standardization. Scientific methods never should be carved in stone, but systematic experimental protocols and uniform methods of data collection and reporting are essential before a second need can be addressed: validation of the assays by careful, comparative studies among laboratories. Baseline frequencies for aneuploidy must be established and the response of each system to a series of known positive compounds assessed. Identification of known positive compounds for aneuploidy induction thus becomes another need. Some reasonable candidates are emerging (e.g., Colcemid in Drosophila and cultured cells, p-fluorophenylalanine in fungi), but much remains to be done. Because there are many potential target sites in the mitotic or meiotic apparatus, several positive control compounds affecting different targets may be necessary.

Technical improvements in the speed, reliability, and accuracy of analysis of existing assay systems undoubtedly will result from efforts to standardize methods. New assay systems, particularly mammalian systems, are also needed. Generation of an extensive database of aneuploidy test results will contribute not only to risk assessment, but to a better understanding of the mechanisms of chromosome segregation. In turn, new insights will provide new ways to test for aneuploidy, either spontaneous or induced by therapeutic or environmental agents.

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DISCUSSION

ÖNFELT: I would like to make a point here. We may also be very interested in identifying new compounds that have a specific target. From my experience, most compounds in vitro, like the mammalian cell culture system, will induce spindle disturbances. I think the C-mitotic effect or colchicine-like disturbances are closely connected with the induction of aneuploidy or polyploidy. Most compounds you introduce can give rise to disturbances and aneuploidy. I would like to stress that toxicity in relation to spindle disturbances is very important. For example, you can make a saturated water solution and treat with compounds, e.g., chloroform, tetrachloroethylene, trichloroethylene, and find an effect both on spindle disturbances and on aneuploidy in vitro. These results may not be relevant to the in vivo situation in one context, because you also observe toxicity in vitro; thus, when comparing results of these chlorinated, aliphatic compounds, they can be very different from the in vivo situation. Some of them are much less toxic in cells in culture in which the cells cannot transform compounds with a mixed function oxidase system. So this enzyme system is extremely important. These compounds that are volatile may also have quite another threshold in vivo (if we accept this reasoning with the threshold), at which the compound can enter the cell directly from a gaseous state. It is a very different situation compared to the compound that enters the cell from a water solution. There are completely different requirements of energy and so on. So the compounds may be orders of magnitude more efficient when they can enter directly from a gaseous state.

GALLOWAY: As one of the Aneuploidy Data Review Committee, I would like to add something to what Dr. Dellarco said this morning. It is really a plea for future test development, and for classifying results the way Dr. Waters has done. We should not just call things positive, negative, or inconclusive. We should somehow build into the call a factor for toxicity. This also reinforces what Dr. Onfelt said which is that you can get nonspecific effects as a result of other methods of toxicity. This also leads to my second point which is if you see something, for example, like a C-mitotic effect in vitro, do not just stop there. Go on and establish that either aneuploid cells result from the exposure, or that they are just a nonspecific effect. I would really like to see toxicity taken into account much more in all these reviews and in everybody's data.

WATERS: If we encounter extreme toxicity in a test system, we always back off a dose. Of course, what is extreme toxicity? That varies from test system to test system. But, in general, we stayed away from toxic phenomena.

ASHLEY: I would like to make a comment regarding cell cycle kinetics. This is something about which many people are not possibly aware. There is a report in the botanical literature that colchicine alters the cell cycle time. There is a considerable delay in the cell cycle when colchicine is added to the system. So this might be something that people will want to take into consideration in planning experiments.

BRINKLEY: Yes, this is the point that I wanted to bring up as well: the cell cycle requires microtubules in G_1--S--G_2. Thus, there are events going on that colchicine could affect.

GAULDEN: We have very definitive, published data on the effects of colchicine on the cell cycle. I would like to emphasize that this is an extremely important aspect.

FORD: When we started looking at flexion of chromosomes as a measure of their tension on attachment of the spindle, we found, much to our alarm, that we were able to induce that effect with sodium chloride. So I think that there should be a word of caution here. Physiological conditions must be maintained very rigorously. For example, we have found that slight changes of temperature could induce massive displacement of chromosomes. I think that these sorts of things have to be looked at very carefully. Another point I would like to make is that chromosome loss should not be regarded as an artifact. I think that there is a lot of evidence to show that it could be a highly repeatable and meaningful phenomenon, that it is probably differently induced to the nondisjunction equivalent of one gain to one loss, and that we could learn a great deal about the action of different agents if we were to score both for hypodiploidy and hyperdiploidy and see how they are related to one another.

SPECIAL COMMITTEE REPORT, PART II: QUANTITATIVE EVALUATION OF CHEMICALS THAT INDUCE ANEUPLOIDY USING THE GENETIC ACTIVITY PROFILE METHOD*

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INTRODUCTION

The qualitative aspects of the Aneuploidy Data Base*** have been summarized in an earlier chapter (Dellarco et al., this Volume). The purpose of this chapter is to address quantitative aspects of the data using the genetic activity profile method described elsewhere (18).

In the present analysis, dose information was obtained from the Aneuploidy Data Base and verified by reference to the original publications. Either the lowest effective dose tested (LEDT) or the highest ineffective dose tested (HIDT) was recorded for each agent and bioassay system. For each compound, the quantitative results in the test systems were represented by computer-generated bar graphs (genetic activity profiles). The x-axis unit values correspond to the various test systems, and the y-axis values are the logarithmically transformed LEDT or HIDT values.

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* The research described in this chapter has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. Reprint requests should be addressed to Michael D. Waters at the above address.

** Operated by Martin Marietta Energy Systems, Inc. under contract No. DE-AC05-80R 21400 with the U.S. Department of Energy.

*** Maintained at the Environmental Mutagen, Carcinogen, and Teratogen Information Program, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

Determination of Effective Doses

For negative test results, the highest dose studied was termed the highest ineffective dose tested (HIDT). If there was evidence of extreme toxicity, the next highest dose was used. A single dose tested with a negative result was considered equivalent to the HIDT. Similarly, for positive results, the lowest effective dose tested (LEDT) was recorded. If probability values were reported, the dose for positive effects was extracted from tables or graphs for which the results were significant at $P \leq 0.05$. If the probability value was not available, the reviewers made a conservative judgment of the dose required for the effect. A clear positive response was indicated when an increase in effect directly proportional to the increase in dose was observed for 2 or more doses; the lower of these doses was generally taken as the LEDT. A single dose was considered equivalent to the LEDT if the observed response was at least twice the spontaneous background.

Dose information in the literature on aneuploidy was reported in various units (e.g., parts per million, percentage, mass per volume, volume per volume). Units for liquids and solids were converted to mass per volume. Concentrations for gaseous compounds, such as halothane, were reported as parts per million on a volume per volume basis. These values were converted to parts per million, mass per volume, according to the ideal gas laws. Dose units for in vitro test systems were expressed as micrograms per milliliter. A volume of 2 ml was assumed for the top agar in microbial plate incorporation assays. (No correction was made for diffusion of the compound into the bottom agar.) Doses for in vivo bioassays are expressed as milligrams per kilogram body weight of the treated animal.

Because of the wide range of doses encountered in bioassays evaluated, the logarithm of the dose was used in calculations and graphs. Because genetic activity was plotted on a log scale, differences in molecular weights of compounds did not greatly influence comparisons of their overall responses.

Representation of a Genetic Activity Profile

A data set consisting of a discrete number of bioassays and quantitative values for the response as a function of dose can be easily represented in a bar graph. This was accomplished for each chemical in the Aneuploidy Data Base by using x-axis unit values corresponding to the various test systems and y-axis values corresponding to the response function for each individual test system. Thus, a discrete array of tests and responses defined a readily interpretable profile of genetic activity.

The sequence of bioassay systems plotted on the x-axis and corresponding bioassay codes and definitions are shown in Tab. 1. The bioassays are grouped according to the phylogenetic classes of test systems. The organization of assay codes is illustrated in Tab. 2. Where appropriate, phylogenetic categories have been separated into mitotic and meiotic assay systems. It may be noted that in Saccharomyces, Aspergillus, Neurospora, and in Drosophila the induction of aneuploidy is detected by genetic tests; whereas in plant and mammalian systems, cytogenetic techniques are used to detect whole chromosome gain or loss.

Tab. 1. Assay codes for aneuploidy tests.

A. Saccharomyces

SC1 = chromosome gain
SC4 = chromosome loss

B. Aspergillus

A1* = euploid segregants - 3 types
A2* = euploid segregants - 1 or 2 types
A3* = euploid segregants - altered ratios
A4* = aneuploid products identified
A5* = aneuploid products inferred

C. Neurospora

NC1 = disomics

D. Plants

PL1 = Allium - somatic
PL2 = Vicia - somatic
PL3 = Hordeum - somatic
PL6 = Pennisetum
PL7 = other

E. Drosophila

DM1 = chromosome loss
DM2 = chromosome gain
DM3 = postmeiotic - chromosome gain
DM4 = loss - stage unspecified
DM5 = gain - stage unspecified

F. Mammalian Cells in Culture

MC1 = rodent - primary - numerical
MC3 = rodent - transformed - numerical
MC5 = human - primary - numerical
MC9 = other

G. Mammalian Somatic Cells In Vivo

MS1 = lymphocytes - numerical
MS3 = rodent - lymphocytes - numerical
MS5 = bone marrow - numerical
MS7 = other

H. Mammalian Female Germ Cells In Vivo

MF1 = M2 oocytes
MF2 = pronuclei
MF3 = preimplantation embryos
MF4 = postimplantation embryos
MF5 = F1 progeny

I. Mammalian Male Germ Cells In Vivo

MM1 = premeiotic - numerical
MM2 = premeiotic - related
MM5 = meiotic - related
MM6 = postmeiotic - related
MM7 = other

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* The last character of the Aspergillus assay code indicates primary or secondary effects: 0 = N/A; 1 = primary; 2 = secondary; 3 = either or both.

Profile line height (the magnitude of each bar) is a function of the LEDT or HIDT, which is associated with characteristics of each individual test system, such as population size, cell-cycle kinetics, and metabolic competency. Thus, the detection limit of each test system is different, and across a given profile, responses will vary substantially. The profiles may be compared and analyzed only on the basis of common test results (i.e., where the same test systems were used to evaluate 2 or more of the chemicals under consideration). No effort was made to adjust or relate responses in one test system to those of another. Such adjustments are difficult unless directly comparable protocols are employed.

To accommodate both positive and negative responses on a continuous scale, doses were transformed logarithmically so that effective (LEDT) and ineffective (HIDT) doses were represented by positive and negative numbers, respectively. The response or logarithmic dose unit (LDU_{ij}) for a given test system i and chemical j was represented by the expressions:

$$LDU_{ij} = -\log_{10}(\text{dose}), \text{ for HIDT values; } LDU \leq 0$$

$$LDU_{ij} = -\log_{10}(\text{dose} \times 10^{-5}), \text{ for LEDT values; } LDU \geq 0$$

These simple relationships define a dose range of 0 to -5 logarithmic units for ineffective doses (1-100,000 μg/ml or mg/kg) and 0 to +7 logarithmic units for effective doses (100,000-0.01 μg/ml or mg/kg). A scale illustrating the LDU values is shown in Fig. 1. Negative responses at doses

Tab. 2. Organization of assay codes for aneuploidy tests.

Mitotic Test Systems	Meiotic Test Systems
A. Saccharomyces (genetic)	
SC4 = chromosome loss	SC1 = chromosome gain
B. Aspergillus (genetic)	
A1* = euploid segregants - 3 types	
A2* = euploid segregants - 1 or 2 types	
A3* = euploid segregants - altered ratios	
A4* = aneuploid products identified	
A5* = aneuploid products inferred	
C. Neurospora (genetic)	
	NC1 = disomics
D. Plants (cytogenetic)	
PL1 = Allium - somatic	
PL2 = Vicia - somatic	
PL3 = Hordeum - somatic	
PL7 = other	
E. Drosophila (genetic)	
DM3 = postmeiotic - chromosome gain	DM1 = chromosome loss
DM4 = loss - stage unspecified	DM2 = chromosome gain
DM5 = gain - stage unspecified	
F. Mammalian Cells in Culture (cytogenetic)	H. Mammalian Female Germ Cells In Vivo (cytogenetic)
MC1 = rodent - primary - numerical	MF1 = M2 oocytes
MC3 = rodent - transformed - numerical	MF2 = pronuclei
MC5 = human - primary - numerical	MF3 = preimplantation embryos
MC9 = other	MF4 = postimplantation embryos
G. Mammalian Somatic Cells In Vivo (cytogenetic)	MF5 = F1 progeny
MS1 = lymphocytes - numerical	I. Mammalian Male Germ Cells In Vivo (cytogenetic)
MS3 = rodent - lymphocytes - numerical	
MS5 = bone marrow - numerical	
	MM1 = premeiotic - numerical
	MM2 = premeiotic - related
	MM5 = meiotic - related
	MM6 = postmeiotic - related
	MM7 = other

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* The last character of the Aspergillus assay code indicated primary or secondary effects: 0 = N/A; 1 = primary; 2 = secondary; 3 = either or both.

less than 1 were set equal to 1. In effect, an LEDT value ≥ 100,000 or an HIDT value ≤1 produces an LDU = 0; therefore, no useful information is gained from such extreme values.

Data Management and Analysis

A computerized data-entry program on disk files was used to store abstracted data from the literature citations. A random file was created for each test system, and a record within this file consisted of a 67-character-array code for chemical number, dose, qualitative, test code, citation number, and additional information. An abbreviated citation corresponding to each publication was stored in an additional file. The data could be searched or sorted for any specified segment of the character arrays by accessing both the test data file and the citation file. The LDU was calculated by searching for the segment containing the dose and calcu-

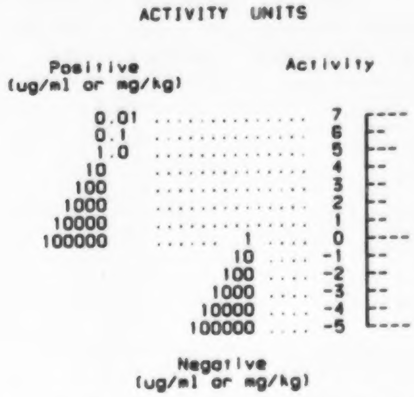


Fig. 1. Scale of logarithmic dose unit values.

lating the appropriate logarithmic function. The LDUs for the set of bioassays for induction of aneuploidy were calculated and stored on magnetic tape in a separate file for each chemical. When conflicting results for a given bioassay were encountered, the subset of data corresponding to the majority of the test results (+ or -) was used. Only those positive or negative results accompanied by a dose value were used to calculate the magnitude of the spectral line. An average of the logarithmic values of the data subset was calculated. In the few cases where numbers of positive and negative results were equal, the overall result for the compound was considered positive, and the doses corresponding to the positive results were used in the calculation.

EVALUATION OF COMPOUNDS TESTED FOR INDUCTION OF ANEUPLOIDY

The performance of assay systems was evaluated initially on a compound-by-compound basis. Compounds for which at least 3 different tests had been performed were selected for graphic presentation. In order to increase the database on vinblastine, information on the free agent was combined with that for vinblastine sulfate. Those compounds for which there were at least 3 different test results are ranked in most-to-least-tested order in Tab. 3 together with their Chemical Abstract Services (CAS) Registry Numbers. The following is a descriptive assessment of the quantitative data on each of these compounds. Each assessment is accompanied by a computer-generated profile and a coded listing of test results and short citation. The test result or call is shown without/with exogenous metabolic activation. Dose values have been converted to μg/ml for in vitro systems or mg/kg for in vivo systems.

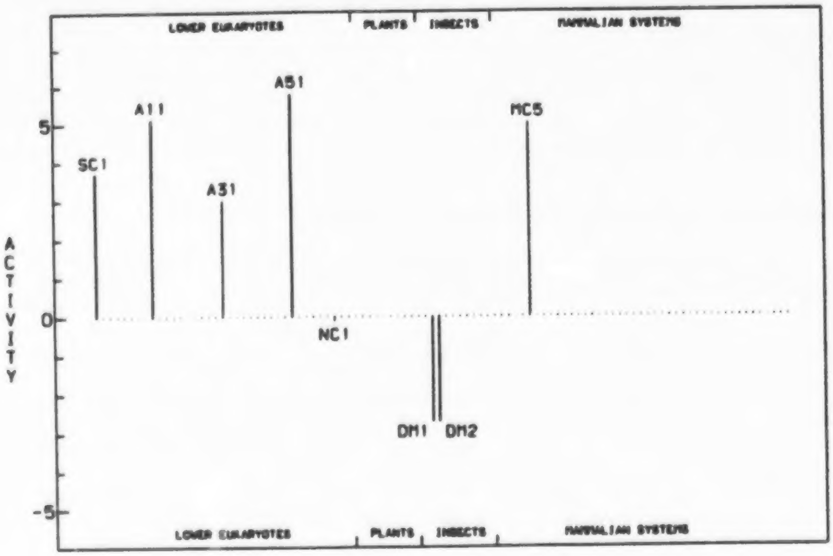
Benomyl

Benomyl is a carbamate that has been widely used as a systemic foliar fungicide. The compound displays very low acute toxicity (>10,000 mg/kg) by the oral route in mammals. In terms of test data acceptable by the Review Committee, benomyl was the most fully evaluated compound.

Benomyl displayed positive results (Fig. 2) for chromosome gain in a meiotic assay in Saccharomyces. The induction of malsegregants was observed in mitotic cells of Aspergillus in 3 different tests. Despite the

Tab. 3. Aneuploidy database.

Chemicals (3 or more tests)	CAS Registry numbers	Number of tests
Benomyl	17804-35-2	8
Cyclophosphamide	50-18-0	6
Triaziquone	68-76-8	6
Amitrole	61-82-5	4
Colchicine	64-86-8	4
Ethanol	64-17-5	4
Vinblastine and Sulfate	865-21-4, 143-67-9	4
Actinomycin D	50-76-0	3
Benzo(a)pyrene	50-32-8	3
Bleomycin	11056-06-7	3
Caffeine	58-08-2	3
Chloral hydrate	302-17-0	3
Colcemid	477-30-5	3
DMSO	67-68-5	3
5-Fluorodeoxyuridine	50-91-9	3
p-Fluorophenylalanine	60-17-3	3
Griseofulvin	126-07-8	3
Halothane	151-67-7	3
Methyl methanesulfonate	66-27-3	3
Simazine	122-34-9	3



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CHEM# TEST CALL    DOSE REF #   REFERENCE

00006 SC1  +/0       20 A0560  SURA ET AL. 17, 1982
00006 A11  +/0     0.80 A0074  MORPURGO ET AL. 81, 1979
00006 A31  +/0      100 A0071  DE BERTOLDI & GRISELLI 303, 1980
00006 A51  +/0     0.15 A0073  KAPPAS 189, 1978
00006 A51  +/0     0.20 A0072  BIGNAMI ET AL. 395, 1977
00006 NC1  -/0        1 A0546  GRIFFITHS 1980
00006 DM1  -/0      500 A0359  LAMB & LILLY 83, 1980
00006 DM2  -/0      500 A0360  LAMB & LILLY 83, 1980
00006 MC5  +/0        1 A0060  TENCHINI ET AL. 139, 1983
00006 MC5  -/0    20000 A0043  GUPTA & LEGATOR 1976
```

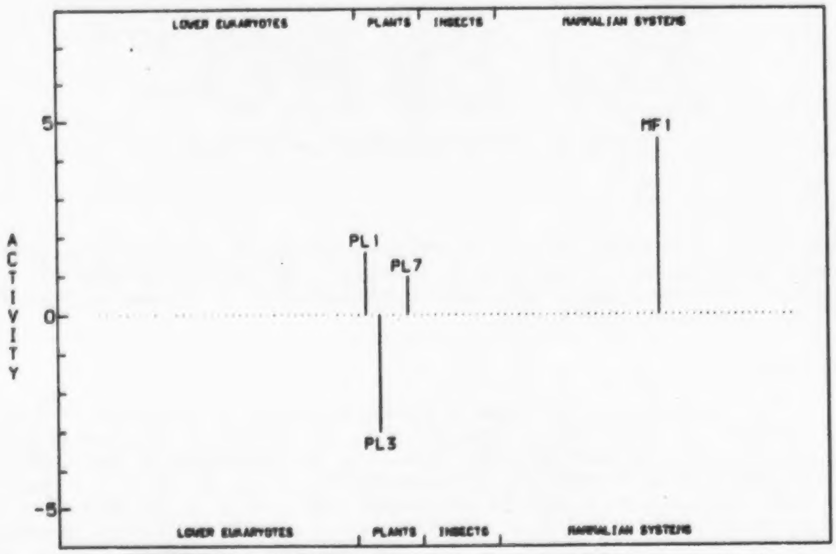
Fig. 2. Activity profile for benomyl.

foregoing positive data, results were negative in a Neurospora meiotic assay for disomics. It should be noted, however, that the test in Neurospora was performed at a relatively low dose level (1 μg/ml). Meiotic assays for both chromosome loss and chromosome gain in Drosophila were negative at high dose equivalents (500 μg/ml). Conflicting positive and negative results were observed in mammalian cells in culture. The positive response (Fig. 2) was observed at a concentration equivalent of 1 μg/ml; however, a negative response was obtained with testing to a high concentration equivalent (20,000 μg/ml).

It should be pointed out that benomyl is frequently found to be converted to or contaminated with its breakdown product, methylbenzimidazole carbamate (MBC). This latter compound also has been shown to induce aneuploidy in 2 test systems (chromosome loss in a mitotic assay in Saccharomyces and induction of malsegregants in a mitotic assay in Aspergillus) (30,62,64).

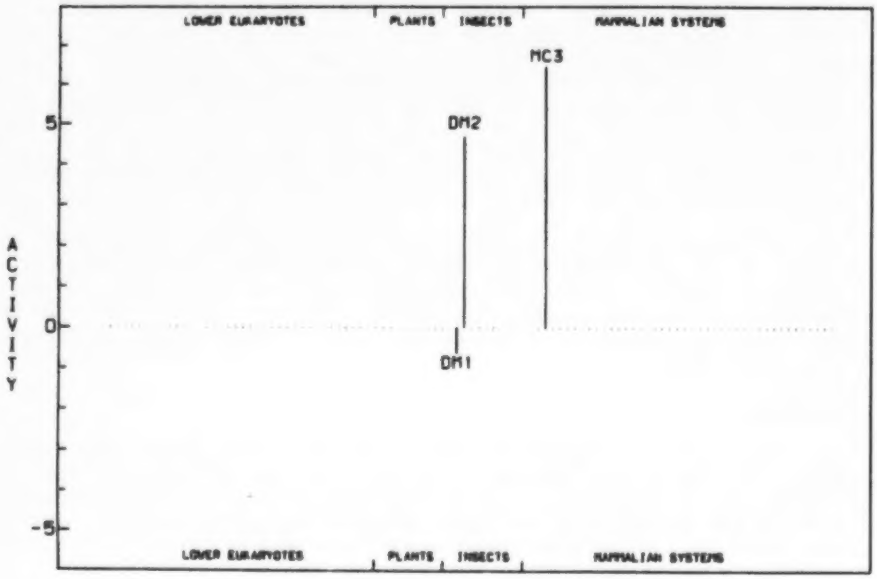
Colchicine

This alkaloid compound has been used medically in the treatment of gout. In view of its classical role as a spindle inhibitor in many test systems, it is surprising that the quantitative database (Fig. 3) acceptable to the Committee is extremely meager. And in plants, in fact, the data conflict. Somatic cell assays in Allium and in Crepis gave positive



CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00012	PL1	+/0	2500	A0635	DAVIDSON ET AL. 437, 1983
00012	PL3	-/0	1000	A0640	SCHWAMMENHOFEROVA & ONDREJ 464, 1978
00012	PL7	+/0	5000	A0636	SAKHAROV ET AL. 69, 1969
00012	PL7	+/0	5000	A0638	PLATONOVA 1283, 1968
00012	PL7	+/0	50000	A0637	PLATONOVA & SVAKINSKAYA 179, 1980
00012	MF1	+/0	3	A0012	SUGAWARA & MIKAMO 251, 1980

Fig. 3. Activity profile for colchicine.



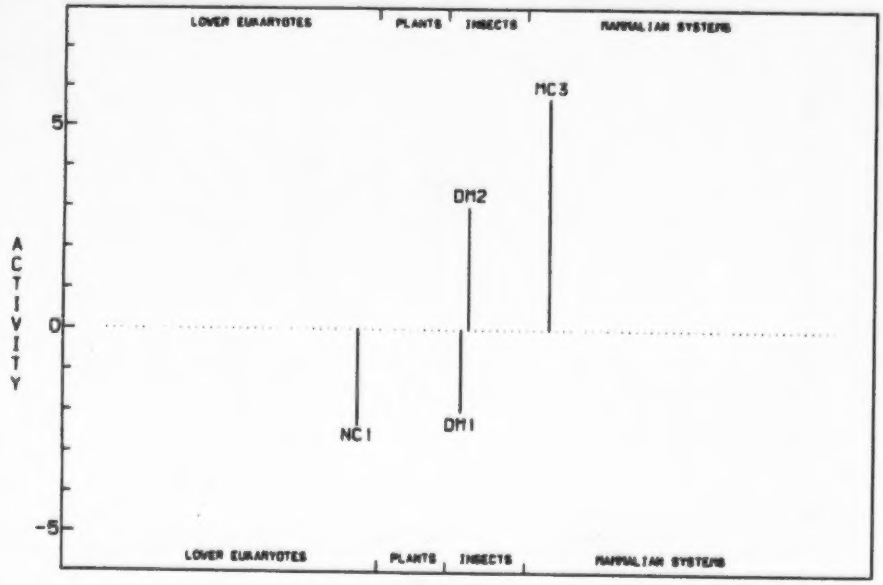
CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00011	DM1	-/0	4.0	A0678	HELD 87, 1982
00011	DM2	+/0	2.0	A0390	HELD 87, 1982
00011	MC3	+/0	0.02	A0188	KURNIN & STAVROVSKAYA 221, 1975
00011	MC3	+/0	0.05	A0054	KATO & YOSHIDA 459, 1970
00011	MC3	+/0	0.05	A0190	HSU ET AL. 201, 1983
00011	MC3	+/0	0.07	A0041	BARASS 85, 1982
00011	MC3	+/0	0.022	A0059	COX ET AL. 18, 1976
00011	MC3	+/0	0.026	A0040	COX 165, 1973

Fig. 4. Activity profile for Colcemid.

results at dose equivalents of 2,500 and 5,000 μg/ml, respectively, while a negative response was observed in Hordium at an equivalent dose of 1,000 μg/ml. Positive effects were seen cytogenetically in metaphase 2 oocytes of Chinese hamsters at 3 μg/ml.

Colcemid

Colcemid is an antineoplastic alkaloid that is structurally similar to colchicine but less toxic in most systems. It also is a well-tested inhibitor of tubulin polymerization. Data (Fig. 4) acceptable by the Committee were obtained in Drosophila or in mammalian cells in culture. Negative results were seen in a meiotic chromosome loss test at a low dose (4 μg/ml) in Drosophila, but a meiotic chromosome gain assay was positive; an overall positive call was recorded by the Subcommittee. Positive results were obtained in several transformed rodent cell systems in vitro at LEDT values of 0.02 to 0.07 μg/ml.

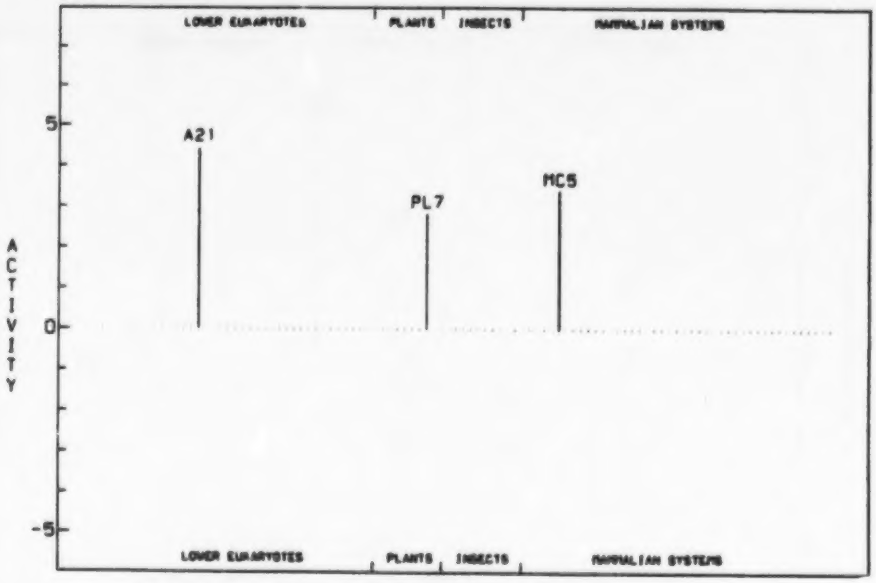


CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00031	NC1	-/0	217	A0156	GRIFFITHS 1980
00031	DM1	-/0	100	A0352	SURSA ET AL. 163, 1980
00031	DM2	+/0	100	A0353	SURSA ET AL. 163, 1980
00031	MC3	+/0	0.8	A0191	HSU ET AL. 201, 1983
00031	MC3	+/0	0.05	A0047	PALYI 307, 1976

Fig. 5. Activity profile for vinblastine and vinblastine sulfate.

Vinblastine and Vinblastine Sulfate

These compounds are also antitumor alkaloids and classic spindle poisons. Results (Fig. 5) in a Neurospora meiotic assay for disomic chromosome gain were negative at 217 µg/ml. In Drosophila, data negative for meiotic chromosome loss, but positive for chromosome gain, were reported at a common dose level of 100 ug/ml. The positive result has been plotted. Numerical chromosome changes were observed at low doses (0.05-0.8 µg/ml) in transformed cells in culture.



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CHEM# TEST CALL    DOSE REF #    REFERENCE

00019 A21  +/0        4 A0085  KAPPAS & GEORGOPOULUS 334, 1974
00019 PL7  +/0      150 A0644  LU SCHIAVO ET AL. 43, 1980
00019 MC5  +/0       40 A0053  LARIZZA ET AL. 123, 1974
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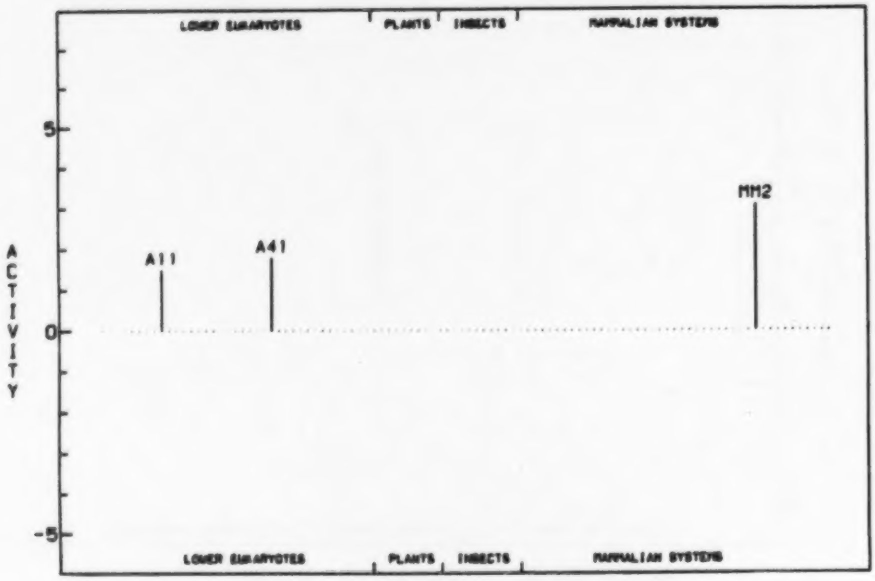
Fig. 6. Activity profile for griseofulvin.

Griseofulvin

Griseofulvin is an antifungal agent used in the treatment of athlete's foot. Positive results (Fig. 6) were obtained in 3 different species: 4 μg/ml is the LEDT in a mitotic test for malsegregants in Aspergillus; at 150 μg/ml, the compound is positive in cultured cells of the plant, Medacago sativa; and mammalian cells in culture display a positive response to the compound at 40 μg/ml.



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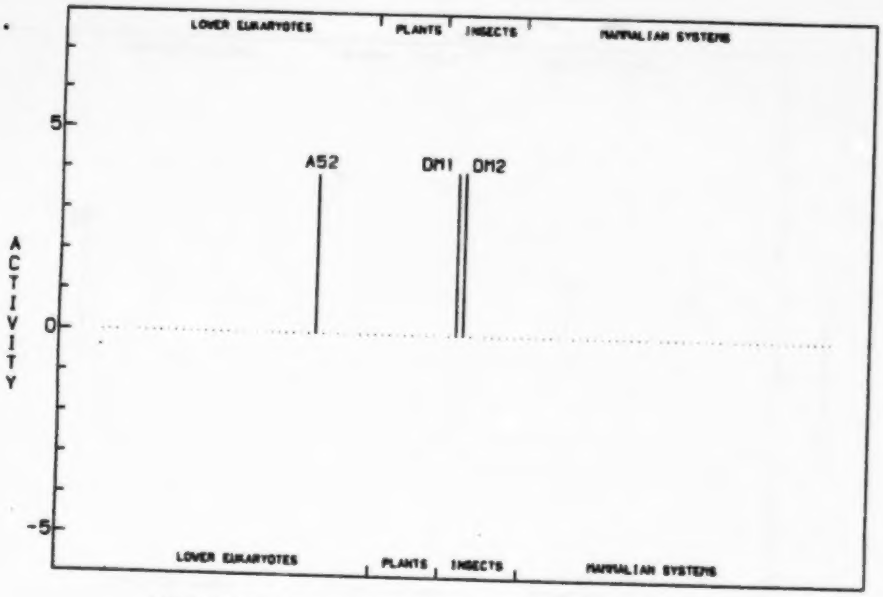
CHEM# TEST CALL     DOSE REF #     REFERENCE

00010 A11   +/0     3300 A0077  SINGH & SINHA 1976
00010 A41   +/0     1650 A0665  KAFER, 1985 (SUBMITTED)
00010 MM2   +/0     82.7 A0661  RUSSO ET AL. 695, 1984
```

Fig. 7. Activity profile for chloral hydrate.

Chloral Hydrate

Chloral hydrate or trichloroacetaldehyde is a metabolite of trichloro- and tetrachloroethylene, both of which are suspect human carcinogens. The compound itself is a sedative and a hypnotic. It appears to block pole-to-pole microtubule formation in addition to other effects on the spindle apparatus. As shown in Fig. 7, at a high concentration (3,300 μg/ml equivalent) in an Aspergillus mitotic test, the compound induces the formation of malsegregants. Aneuploid products have been observed in Aspergillus at a dose of 1,650 μg/ml equivalent. The compound is active in mice (82.7 mg/kg) in a meiotic assay for numerical chromosome changes in male germ cells.



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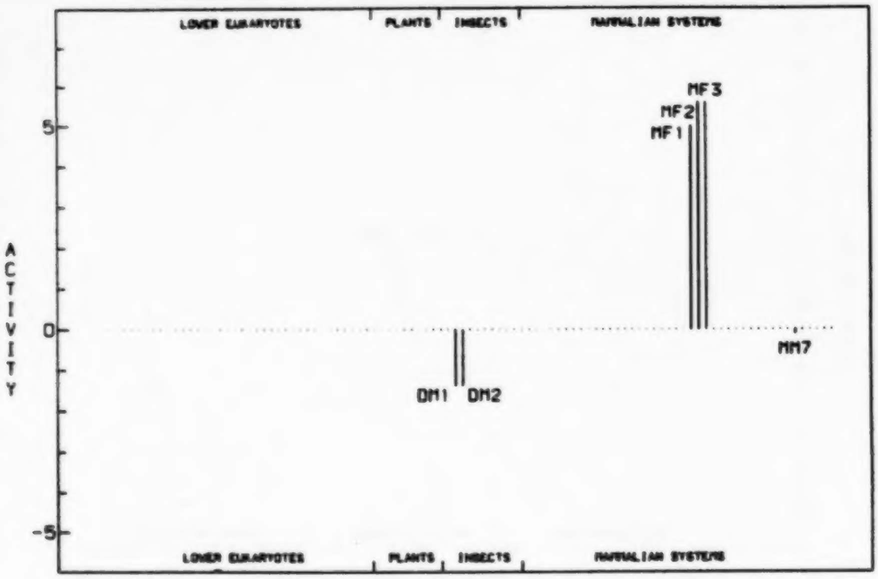
CHEM# TEST CALL    DOSE REF #     REFERENCE

00002 A52  +/0     12.6 A0069  KAPPAS 189. 1978
00002 DM1  +/0       10 A0500  FELIX & RODRIGUEZ 13. 1968
00002 DM2  +/0       10 A0501  FELIX & RODRIGUEZ 13. 1968
```

Fig. 8. Activity profile for actinomycin D.

Actinomycin D

This antibiotic has been used as an antineoplastic agent. The compound directly inhibits nucleic acid synthesis and may secondarily affect centriole duplication. In mitotic assays in Aspergillus (Fig. 8), actinomycin D induces, at 12 µg/ml, the formation of aneuploid products (products inferred). In meiotic assays in Drosophila, the compound induces both chromosome loss and chromosome gain at doses equivalent to 10 µg/ml.

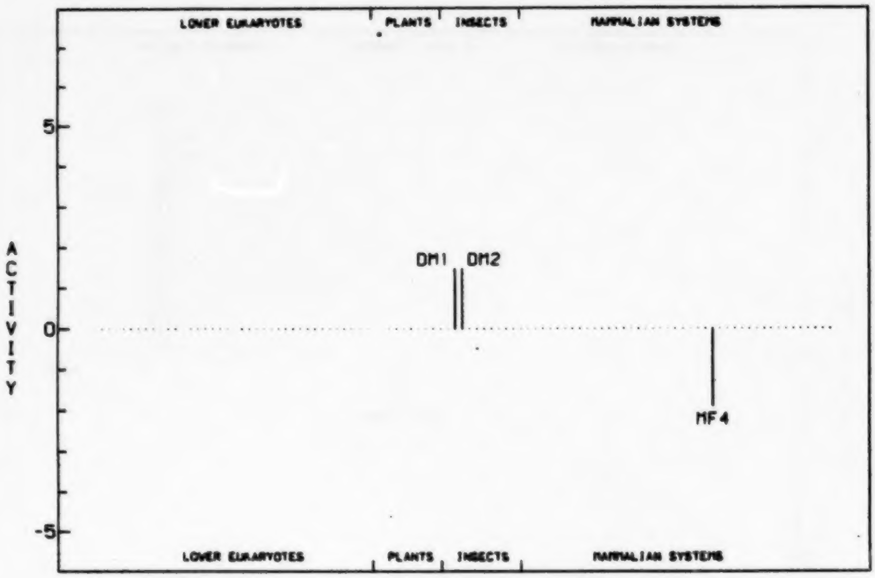


CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00029	DM1	-/0	23.1	A0504	VOGEL 339, 1973
00029	DM2	-/0	23.1	A0505	VOGEL 339, 1973
00029	MF1	+/0	1.0	A0032	BECKER & SCHONEICH 447, 1982
00029	MF1	-/0	2.0	A0031	BASLER 67, 1978
00029	MF2	+/0	0.25	A0033	BECKER & SCHONEICH 447, 1982
00029	MF3	+/0	0.25	A0037	HANSMANN & ROHRBORN 101, 1973
00029	MM7	-/0	0.25	A0567	BINKERT & SCHMID 77, 1977

Fig. 9. Activity profile for triaziquone.

Triaziquone

This aziridine-substituted benzoquinone is a potent gene mutagen and clastogen in mammals. Surprisingly, therefore, it is inactive in Drosophila meiotic assays for chromosome loss and chromosome gain. Positive results in mice and negative results in hamsters (Fig. 9) have been obtained in meiotic metaphase 2 oocytes at low dose equivalents (1.0 and 2.0 mg/kg, respectively). In unfertilized ova in mice, the compound produces positive results at 0.25 mg/kg. Similar results have been obtained in preimplantation embryos in mice. Interestingly, triaziquone gives negative results in hamster male germ cells at 0.25 mg/kg equivalent.



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CHEM# TEST CALL    DOSE REF #    REFERENCE

00020 DM1  +/0     3400 A0366  CLEMENTS & TODD 225, 1981
00020 DM2  +/0     3400 A0367  CLEMENTS & TODD 225, 1981
00020 MF4  -/0       79 A0020  BASLER & ROHRBURN 143, 1981
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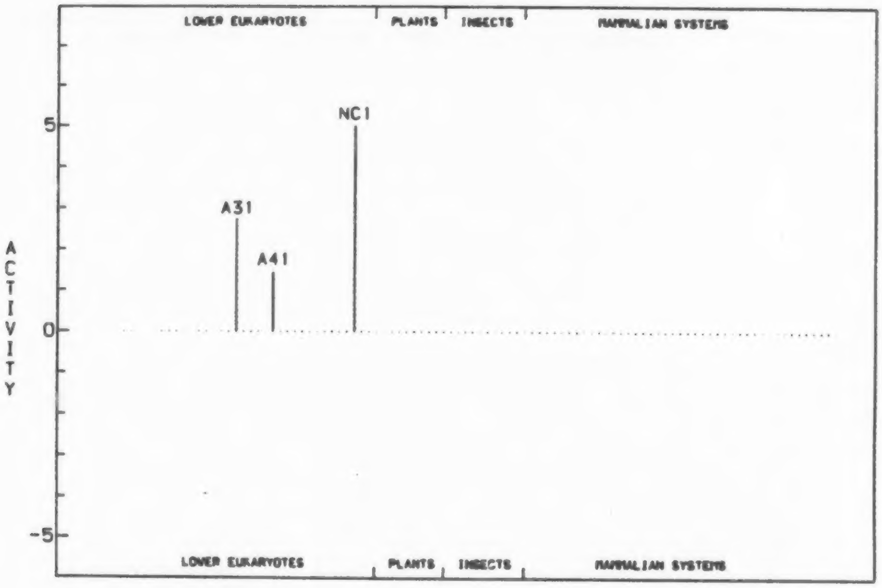
Fig. 10. Activity profile for halothane.

Halothane

Halothane is very widely used as an anesthetic gas in humans. As shown in Fig. 10, it produces positive results for chromosome loss and chromosome gain in meiotic assays when fed to Drosophila at 3,400 μg/ml. On the other hand, an aerosol dose equivalent of 79 μg/ml is negative for meiotic nondisjunction in female mice.

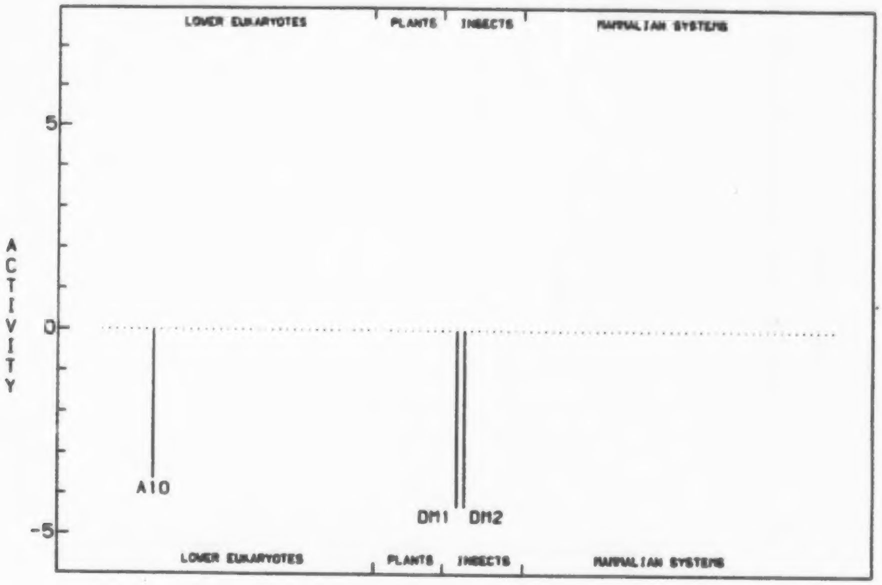


CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00025	SC4	+/0		A0672	STROMNAES 197, 1968
00025	A31	+/0	183	A0093	DE BERTOLDI & GRISELLI 303, 1980
00025	A41	+/0	3660	A0092	SHANFIELD & KAFER 209, 1971
00025	NC1	+/0	1	A0128	GRIFFITHS 1980

Fig. 11. Activity profile for p-fluorophenylalanine.

Para-Fluorophenylalanine

This amino acid analog is incorporated into proteins, thus producing direct and indirect effects on mitotic as well as meiotic systems. It is positive in Saccharomyces at an undefined dose level. As shown in Fig. 11, the compound induces altered ratios of malsegregants and identifiable aneuploid products in Aspergillus at 183 and 3,660 μg/ml, respectively. Disomics are induced in Neurospora at a low dose (1 μg/ml). Para-fluorophenylalanine, in fact, has been used as a positive control in the Neurospora assay.

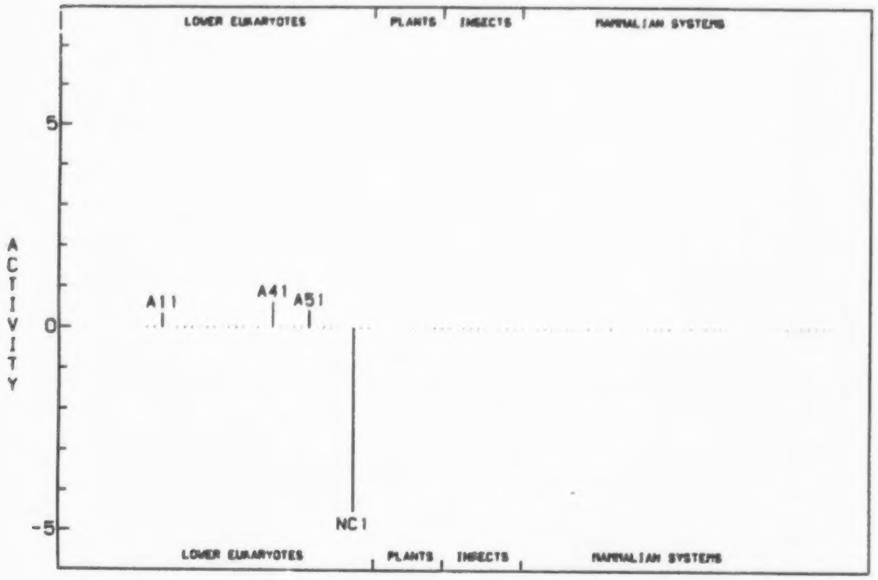


CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00016	A10	-/0	4100	A0667	MORPURGO ET AL. 81, 1979
00016	DM1	-/0	20000	A0413	TRAUT 273, 1983
00016	DM2	-/0	20000	A0414	TRAUT 273, 1983

Fig. 12. Activity profile for dimethyl sulfoxide.

Dimethyl Sulfoxide

Dimethyl sulfoxide (DMSO) is commonly used as a solvent for test agents in short-term, in vitro assay systems. It is most often negative in genetic tests. DMSO (Fig. 12) failed to induce euploid segregants in mitotic assays in Aspergillus (at 4,100 μg/ml), and produced negative results in meiotic chromosome loss and chromosome gain assays in Drosophila at 20,000 μg/ml.

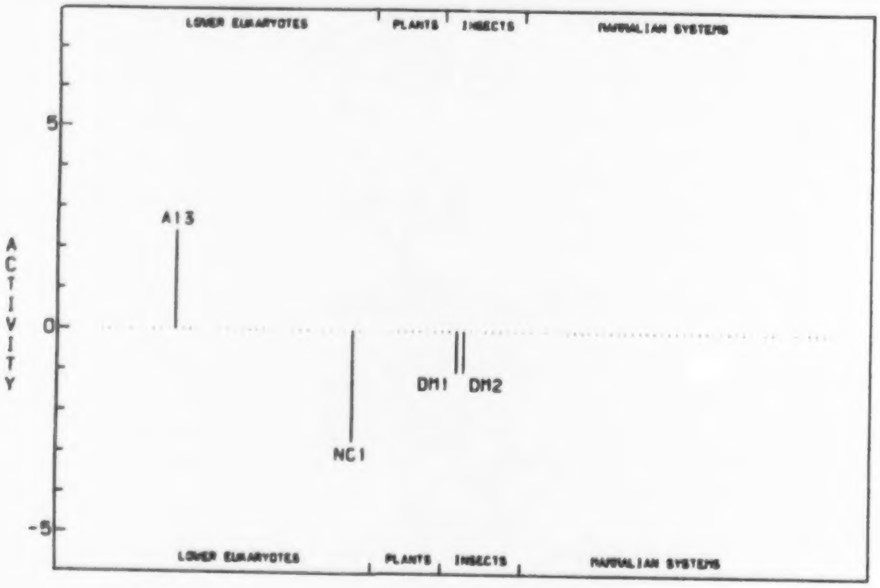


CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00017	A11	+/0	48000	A0080	MURPURGO ET AL. 81, 1979
00017	A41	+/0	24000	A0082	KAFER 53, 1984
00017	A51	+/0	40000	A0081	HARSANYI ET AL. 51, 1977
00017	NC1	-/0	32000	A0132	GRIFFITHS 1980

Fig. 13. Activity profile for ethanol.

Ethanol

Ethanol (Fig. 13) induces malsegregants in 3 mitotic tests in Aspergillus, but only at levels of 24,000 to 40,000 μg/ml. Negative results were obtained in Neurospora at a similar dose (32,000 μg/ml). No reports in mammalian systems were accepted by the Review Committee. There is definitely a need for further studies on this compound in higher eukaryotic test systems.



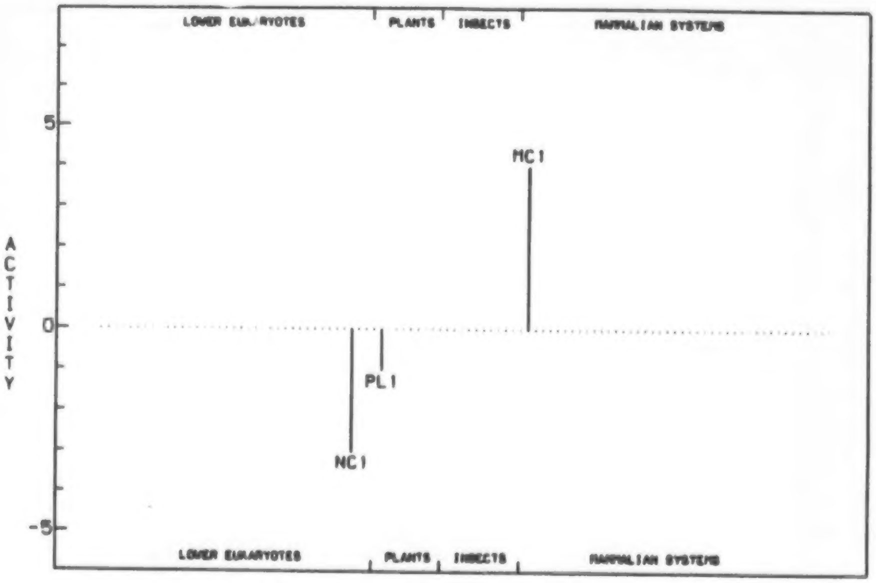
CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00004	A13	(+)/0	400	A0102	BIGNAMI ET AL. 395, 1977
00004	NC1	-/0	500	A0129	GRIFFITHS 1980
00004	DM1	-/0	10	A0305	LAAMENEN ET AL. 185, 1976
00004	DM2	-/0	10	A0306	LAAMENEN ET AL. 185, 1976

Fig. 14. Activity profile for amitrole.

Other Genotoxic Chemicals

Several additional compounds were tested in 3 or more assays with mainly positive results. The following are examples of compounds which are generally genotoxic and may be considered nonspecific inducers of aneuploidy.

Amitrole. Amitrole or 3-amino-S-triazole is used as a herbicide. It displays an acute oral LD_{50} in rats of 1,000-2,500 mg/kg with a dermal LD_{50} of greater than 10,000 mg/kg. The Committee's review revealed (Fig. 14) a weak positive response in Aspergillus for the induction of somatic malsegregants. The positive response was observed at 400 μg/ml. A meiotic assay for disomics in Neurospora gave a negative response at 500 μg/ml. Negative responses were reported for meiotic chromosome loss and chromosome gain in Drosophila of a concentration equivalent of 10 μg/ml.



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CHEM# TEST CALL     DOSE REF #     REFERENCE

00007 NC1   -/0     1000 A0547   GRIFFITHS 1980
00007 PL1   -/0       10 A0632   FISKESJO 155, 1981
00007 MC1   +/0        1 A0193   BENEDICT          588, 1972
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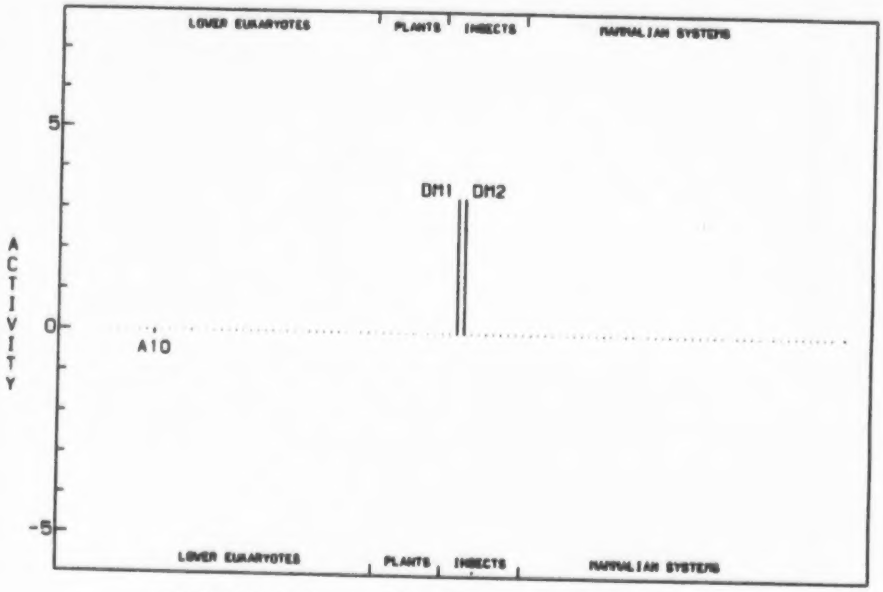
Fig. 15. Activity profile for benzo(a)pyrene.

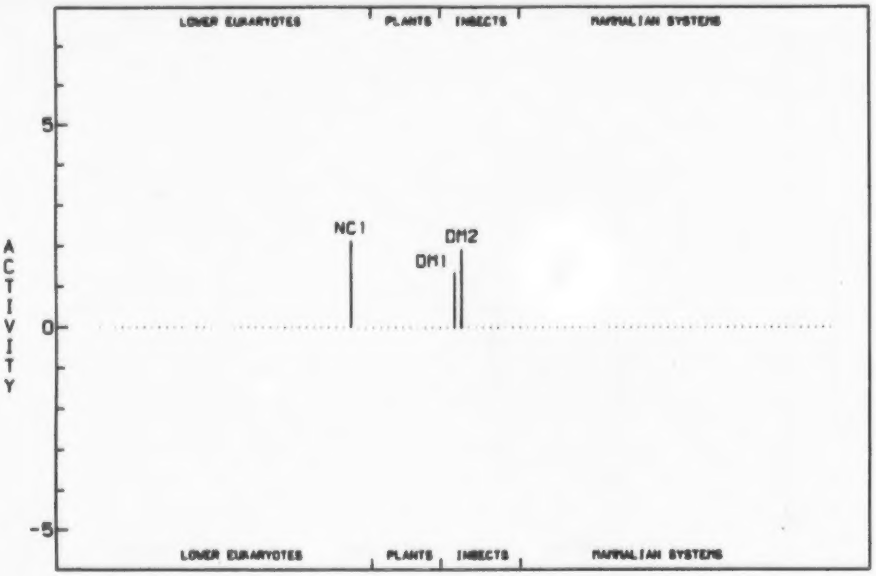
Benzo(a)pyrene. Benzo(a)pyrene (Fig. 15) is an extremely widespread polynuclear aromatic hydrocarbon. It requires metabolic activation and is negative in a nonactivated meiotic disomic assay in Neurospora at a concentration of 1,000 μg/ml. Similarly, the compound is negative in a somatic assay in Allium at a concentration equivalent of 10 μg/ml. Rodent primary cells in culture displayed numerical chromosome changes in the absence of metabolic activation at 10 μg/ml.



CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00008	A10	-/0	0.5	A0075	DEMOPOULOS ET AL. 51, 1982
00008	DM1	+/0	50	A0354	TRAUT 89, 1980
00008	DM2	+/0	50	A0355	TRAUT 89, 1980

Fig. 16. Activity profile for bleomycin.

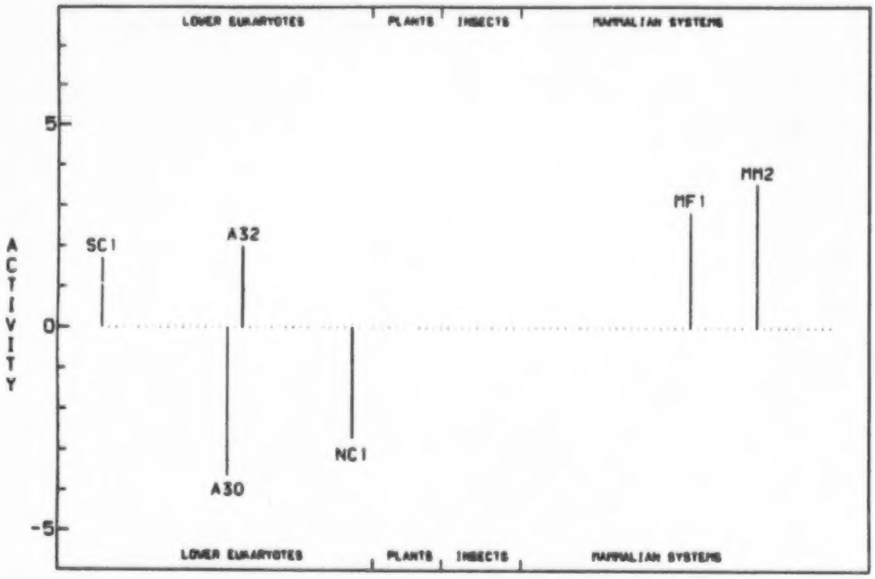
Bleomycin. This antifungal agent (Fig. 16) was negative in Aspergillus for the induction of aneuploidy at a concentration of only 0.5 μg/ml. In Drosophila, the compound was positive in meiotic assays for chromosome loss and chromosome gain.



CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00009	NC1	+/0	750	A0124	GRIFFITHS 1980
00009	DM1	+/0	1940	A0474	CLARK & CLARK 227, 1968
00009	DM1	+/0	5000	A0288	ZETTLE & MURNIK 146, 1973
00009	DM1	+/0	10000	A0491	OSTERTAG & HAAKE 299, 1966
00009	DM1	-/0	1940	A0476	CLARK & CLARK 227, 1968
00009	DM2	+/0	1230	A0488	MITTLER ET AL. 708, 1967B
00009	DM2	-/0	1940	A0475	CLARK & CLARK 227, 1968
00009	DM2	-/0	5000	A0289	ZETTLE & MURNIK 146, 1973
00009	DM2	-/0	10000	A0492	OSTERTAG & HAAKE 299, 1966

Fig. 17. Activity profile for caffeine.

Caffeine. Caffeine (Fig. 17) displayed positive results in a Neurospora meiotic assay for disomics at a concentration of 750 μg/ml. Meiotic assays for chromosome loss in Drosophila were predominantly positive at concentration equivalents ranging from 1,940 to 10,000 μg/ml. Meiotic assays for chromosome gain over the same dose range were predominantly negative. The Drosophila subcommittee considered caffeine positive for chromosome gain in meiotic cells in Drosophila larvae but not in adults.



CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
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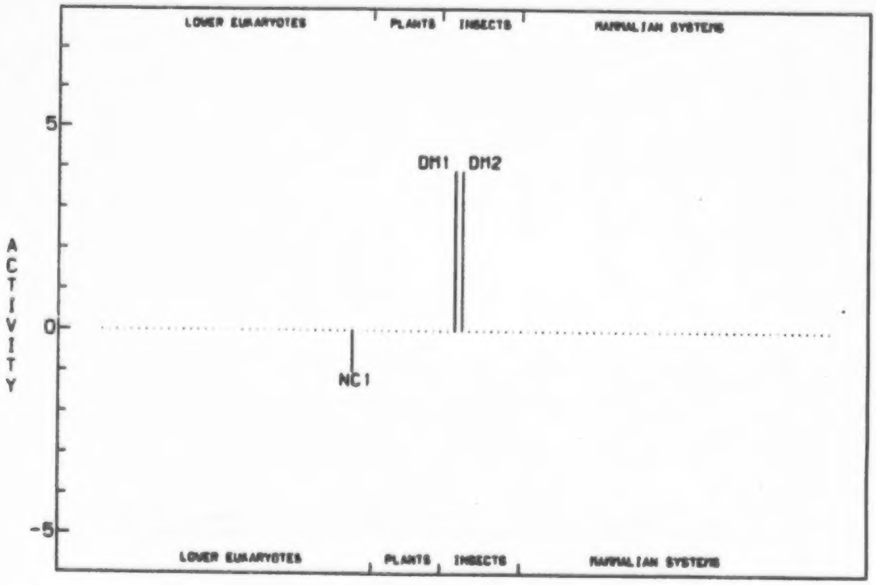
00013	A30	-/0	4180	A0666	DE BERTOLDI & GRISELLI 303, 1980
00013	A32	0/+	1040	A0078	DE BERTOLDI & GRISELLI 303, 1980
00013	NC1	-/0	500	A0528	GRIFFITHS 1980
00013	MF1	+/0	145	A0015	ROHRBORN & HANSMANN 184,1971
00013	MF1	+/0	150	A0017	BEEBER & SCHONEICH 447, 1982
00013	MM2	+/0	30	A0625	PACCHIEROTI ET AL. 177, 1983

Fig. 18. Activity profile for cyclophosphamide.

Cyclophosphamide. Cyclophosphamide (Fig. 18) is a widely used chemotherapeutic agent which is known to require metabolic activation in most short-term genetic bioassays. The compound gave positive results in a meiotic chromosome gain assay in Saccharomyces at a concentration of 2,000 μg/ml in the absence of exogenous metabolic activation. Negative results were obtained in the absence of exogenous metabolic activation in an Aspergillus assay for aneuploid products. Positive results were obtained, however, in the same assay in the presence of an S-9 metabolic activation system. In the absence of metabolic activation, a negative response was obtained in a meiotic assay for disomics in Neurospora. Female (M2 oocytes) and male germ cell assays for induction of aneuploidy were positive at 145-150 mg/kg or 30 mg/kg, respectively.



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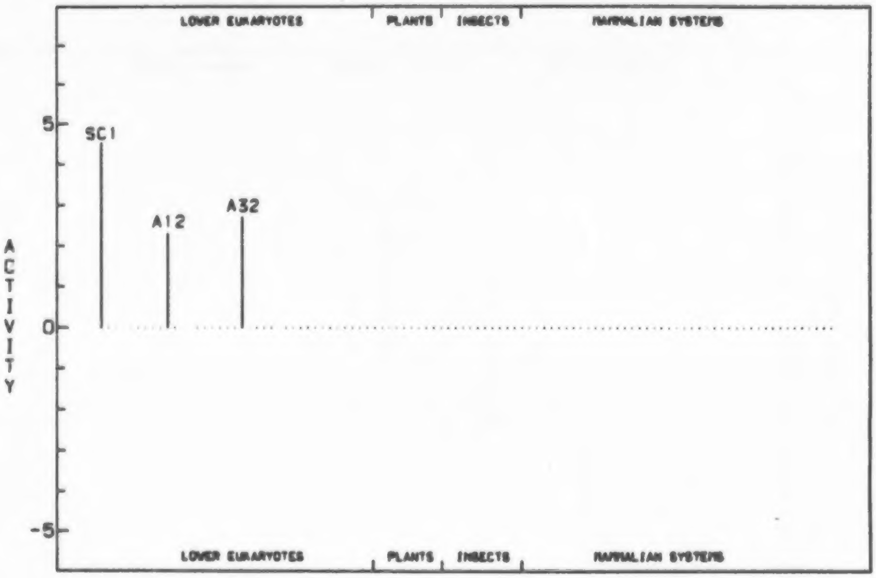
CHEM# TEST CALL      DOSE REF #    REFERENCE

00001 NC1   -/0        10 A0119   GRIFFITHS 1980
00001 DM1   +/0      12.5 A0309   TRAUT 259, 1978
00001 DM2   +/0      12.5 A0310   TRAUT 259, 1978
```

Fig. 19. Activity profile for 5-fluorodeoxyuridine.

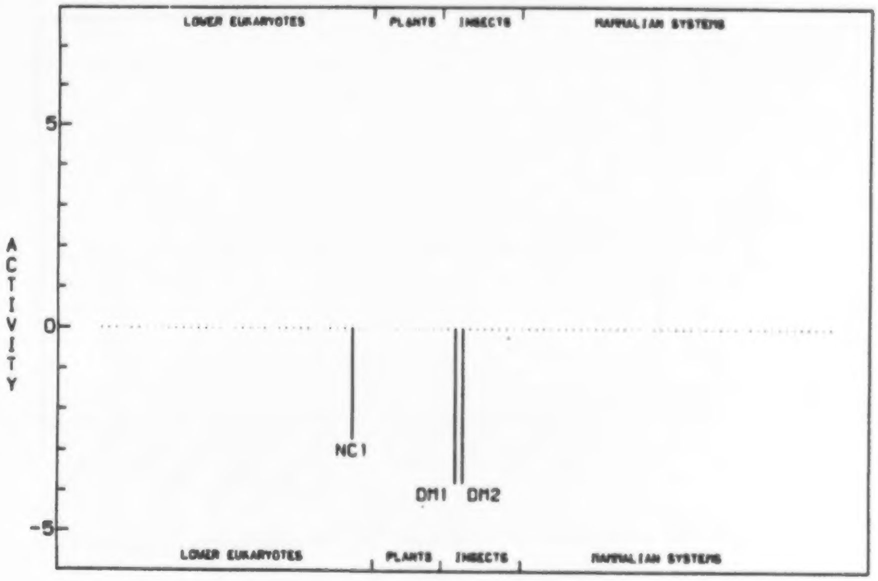
5-Fluorodeoxyuridine. This nucleic acid analog and antitumor agent (Fig. 19) was negative in a meiotic assay for disomics in Neurospora to a relatively low concentration of 10 μg/ml. Meiotic assays in Drosophila for chromosome loss and chromosome gain returned positive responses at dose equivalents of 12.5 μg/ml in both assays.



CHEM#	TEST	CALL	DOSE	REF #	REFERENCE
00022	SC1	+/0	3	A0559	SORA ET AL. 17, 1982
00022	A12	+/0	500	A0087	GUALANDI ET AL. 255, 1979
00022	A32	+/0	200	A0088	DE BERTOLDI & GRISELLI 303, 1980

Fig. 20. Activity profile for methyl methanesulfonate.

Methyl methanesulfonate. This well-known, monofunctional alkylating agent (Fig. 20) gave positive results in a meiotic assay for chromosome gain in Saccharomyces at a dose of only 3 μg/ml. Higher concentrations (200 to 500 μg/ml) were required to induce the appearance of malsegregants in Aspergillus.



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CHEM# TEST CALL     DOSE REF #    REFERENCE

00026 NC1   -/0      500 A0517  GRIFFITHS 1980
00026 DM1   -/0     6000 A0332  MURNIK & NASH 691, 1977
00026 DM2   -/0     6000 A0333  MURNIK & NASH 691, 1977
```

Fig. 21. Activity profile for simazine.

Simazine. Simazine (Fig. 21) is a chlorinated triazine herbicide. It gave negative results in a meiotic assay for disomics in *Neurospora* at a dose of 500 µg/ml. Similarly, negative responses were obtained at dose equivalents of 6,000 µg/ml in meiotic assays for chromosome loss or chromosome gain in Drosophila.

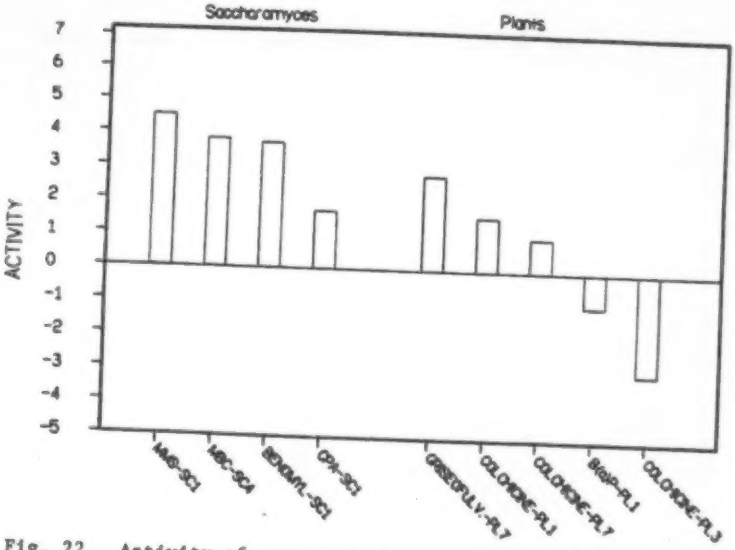

Fig. 22. Activity of compounds in Saccharomyces and plants.

Assessment of Test System Performance by Phylogenetic Class

Lowest effective dose levels may be compared in profiles presented with tests grouped according to their phylogenetic category. For convenience, test results from Saccharomyces and higher plant systems are placed together on the same graph (Fig. 22).

Saccharomyces. This system (Fig. 22) responds to agents, including methyl methanesulfonate and cyclophosphamide, that have been shown to be genotoxic in many other assays. Benomyl and its breakdown product MBC are both positive at approximately equivalent doses (10-20 μg/ml) in Saccharomyces.

Plants. Griseofulvin (Fig. 22) was found positive in cultured cells from *Medicago sativa*. Colchicine gives positive results in Allium and in *Crepis capillaris*, but negative results in Hordium. Since roughly similar dose levels were tested (1,000-5,000 μg/ml), the reason for such discordant results is not immediately apparent. Benzo(a)pyrene gave negative results in Allium.

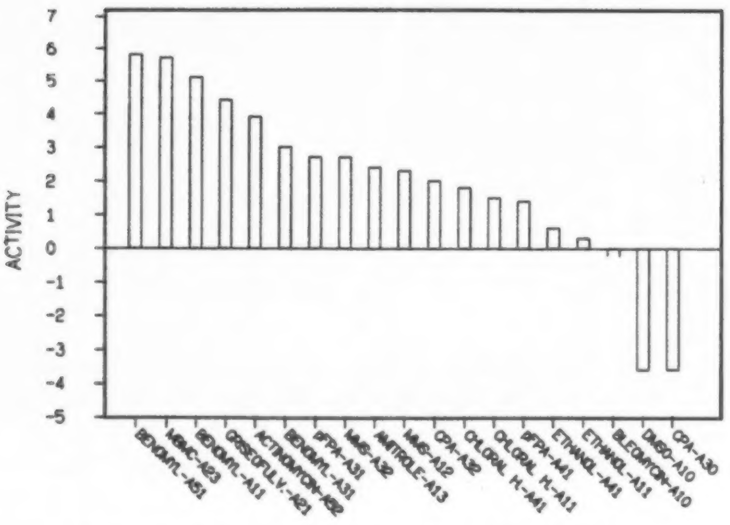

Fig. 23. Activity of compounds in Aspergillus.

Aspergillus. Aspergillus (Fig. 23) responded positively to relatively low concentrations of benomyl (0.15-0.8 µg/ml) and MBC (0.2 µg/ml). Griseofulvin was found positive in Aspergillus at somewhat lower concentrations (4 µg/ml) than in higher plants (150 µg/ml). In general, it appears that alkylating agents, such as MMS and cyclophosphamide, are active in Aspergillus at higher concentrations than are agents such as benomyl, MBC, and griseofulvin. The latter agents may induce aneuploidy in a more direct manner. As mentioned previously, ethanol produces positive results only at very high dose levels and DMSO gives negative responses. The negative result with bleomycin is somewhat unexpected, but may result from the fact that a relatively low dose level (0.5 µg/ml) was tested.

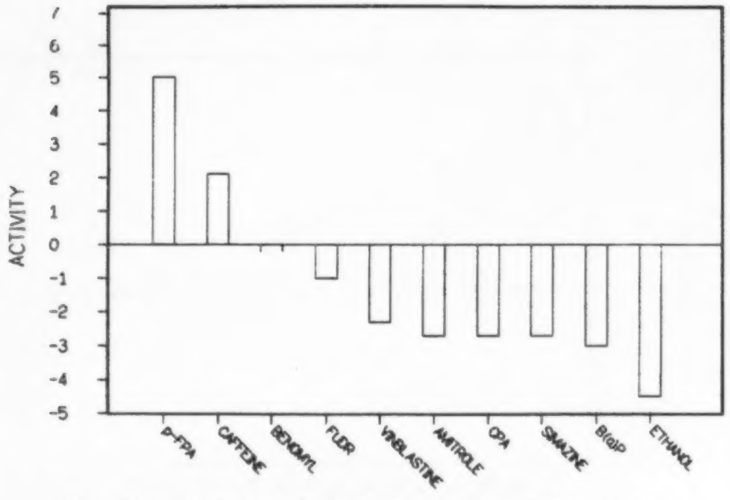

Fig. 24. Activity of chemicals in Neurospora (NC1).

Neurospora. Neurospora (Fig. 24) displays an unusual profile of responses to several chemicals in the meiotic test for disomics. Notable are the negative responses for benomyl, cyclophosphamide, and vinblastine.

Drosophila. In general, meiotic assays for chromosome gain in Drosophila (DM2) represent a classic pattern of responses for the agents represented in Fig. 25. The spindle inhibitor, Colcemid, is active in the system at a relatively low concentration (12 μg/ml). Interestingly, the carbamate fungicide, benomyl, is inactive with moderately high concentrations (500 μg/ml) having been tested. The triazine herbicide, simazine, is negative in Drosophila when tested at 6,000 μg/ml.

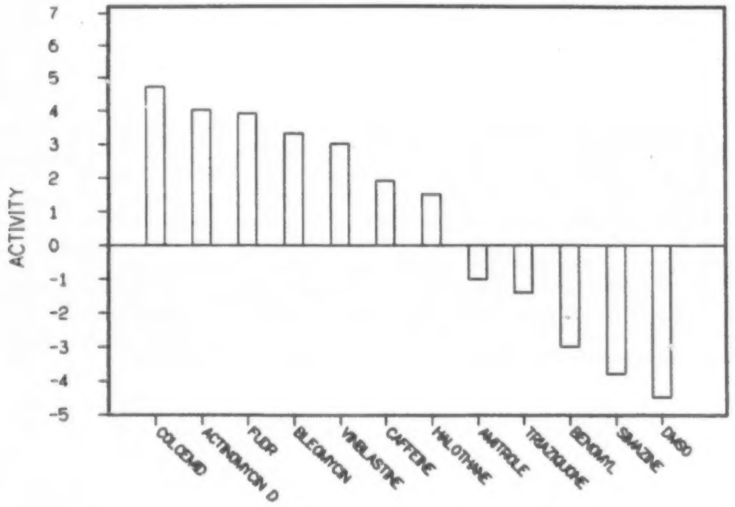

Fig. 25. Activity of chemicals in Drosophila (DM2).

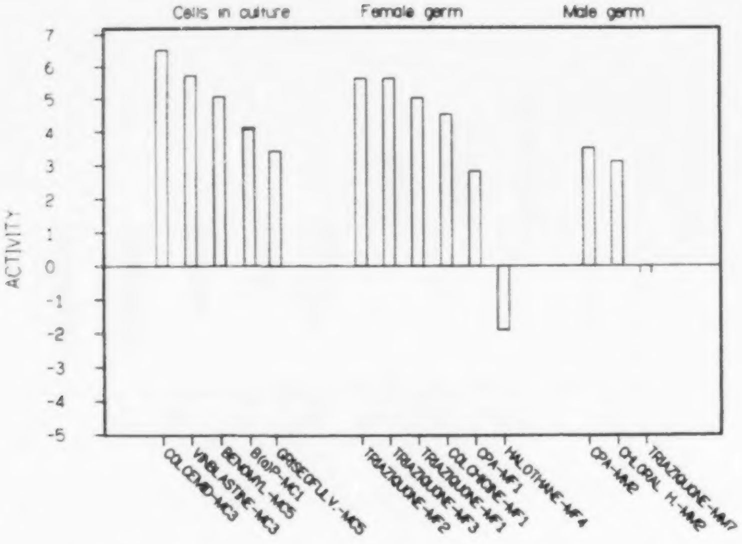

Fig. 26. Activity of chemicals in mammalian systems.

Mammalian systems. Mammalian systems are represented collectively in Fig. 26. Mammalian cells in culture responded positively to low doses of the classical spindle poisons, Colcemid, vinblastine, and benomyl (0.02-1 μg/ml), and to higher doses of griseofulvin (40 μg/ml). Benzo(a)pyrene also induced aneuploidy at 10 μg/ml in mammalian cells in the process of in vitro transformation.

Germ cell studies in mammals are very limited. The positive responses for triaziquone in various female germ cell stages are notable in view of the negative response observed at the same concentration (0.25 mg/kg) in male germ cells. The well-known chemotherapeutic agent, cyclophosphamide, gives positive results in both female and male germ cells. Also, the anesthetic gas, halothane, is negative in female germ cells.

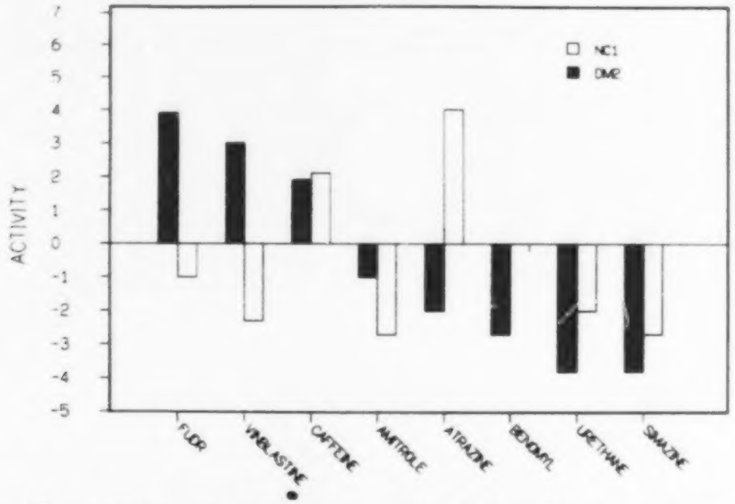

Fig. 27. Activity of compounds tested in Drosophila and Neurospora.

Comparative evaluation of Neurospora and Drosophila. It was possible to compare results for test systems within different phylogenetic classes (Fig. 27) only in the cases of Neurospora (NC1) and Drosophila (DM2). The assays compared were meiotic ones and each represented the gain of chromosomes.

Eight chemicals were evaluated in both of the assays; results for only 5 of these agents were concordant. The only concordantly positive results were reported with caffeine. 5-Fluorodeoxyuridine and vinblastine were positive in Drosophila but negative in Neurospora. Atrazine was negative in Drosophila but positive in Neurospora.

Summary of Positive Data

Mitotic and meiotic test systems may be compared (Fig. 28). Actinomycin D, benomyl, chloral hydrate, Colcemid, colchicine, cyclophosphamide, p-fluorophenylalanine, and vinblastine are reported positive in both mitotic and meiotic assays. Generally, LEDT values are similar for mitotic and meiotic tests in cases where a chemical has been tested in 2 or more phylogenetic classes. Of the 20 compounds with 3 or more test results, DMSO and simazine tested negatively in all systems utilized to measure aneuploidy.

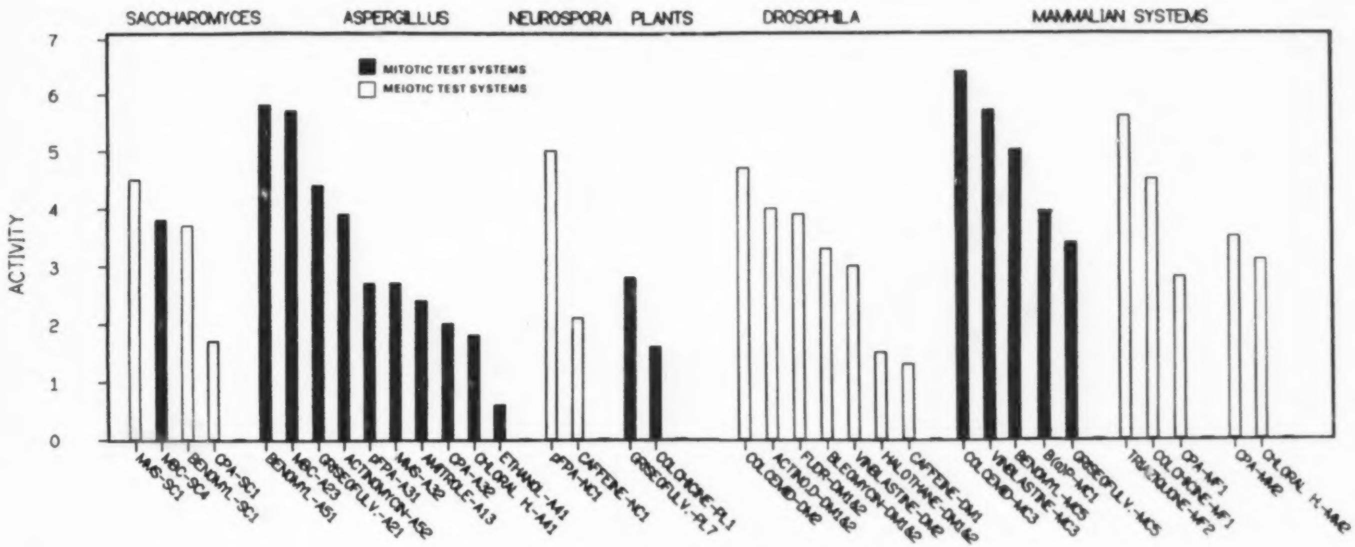

Fig. 28. Summary of positive effects by phylogenetic class.

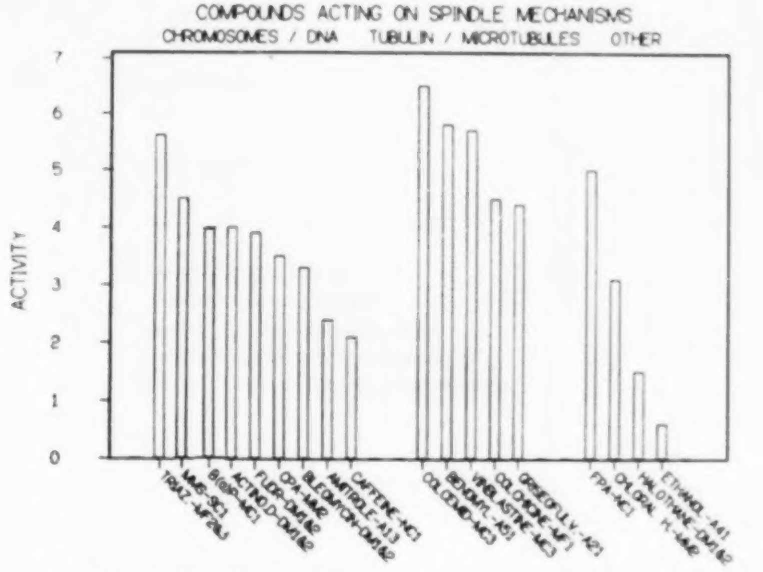

Fig. 29. Classification of compounds based on mechanism of action.

Classification of Compounds Based on Mechanism of Action

In Fig. 29, those compounds displaying results in 3 or more test systems have been placed in rank order by LEDT, and have been grouped according to the classification scheme of Bond and Chandley (8). This scheme subdivides compounds reported to induce aneuploidy into those that: (a) interact with chromosomes and/or DNA; (b) affect tubulin or microtubules; or (c) influence the spindle in other ways. For further discussion on this topic see Bond and Chandley (8) and the following chapter by Liang and Brinkley (this Volume).

CONCLUSION

The foregoing data support the conclusion that there are very few data on the induction of aneuploidy in well-defined test systems. To the extent that protocols can be established, further standardization of test systems is required. Clearly, information is required on test system performance against a common set of test agents. Such agents, ideally, would be understood with regard to their mechanism(s) of action.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the excellent typing assistance of Reta C. Hancock and Carol A. Kuehnhoff.

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DISCUSSION

BOND: I would like to make a point with regard to the process of classifying the results. I think there is a danger here. The process is a decision-making process, and you have to decide what you do when the data are in conflict within a particular test system. For example, with respect to caffeine, there is no doubt that it induces chromosome loss in Drosophila. It also induces binucleate cells and diploidy. But when you come to the issue of chromosome gain in Drosophila, there is one positive test and 5 negatives, I think. Or there are 2 positive reports from one lab, and 5 negatives, or 2 negatives and 3 inconclusives, or something. It is very much more doubtful whether caffeine induces chromosome gain, for example. Then the classification process has to take into account conflicts of that sort. It is worth stressing that there has to be very careful evaluation of the ground rules that have gone into coming up with a conclusion, because people read the conclusions without taking into account the processes by which one arrives at these conclusions.

WATERS: There was one plot on the entire series of graphs that was a committee call and went against the rules that we had established to take a majority call for assay results. So if we had taken our conventional approach there, we would have agreed with you. But the committee called that result positive. We do show the database--the number of test results and the individual test data with and without activation that were required--below each of the plots. Your point is well taken.

BOND: There is the point of loss versus gain. It does promote chromosome loss; it is an aneuploidy inducer in that sense. But perhaps gains are more important for us.

[Unidentified speaker]: I have been working with Dr. Stan Zimmering. We have tested caffeine in a couple other systems and it induces both gain and loss in those systems of Drosophila.

CHEMICAL PROBES AND POSSIBLE TARGETS FOR THE INDUCTION OF ANEUPLOIDY

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ABSTRACT

There is an increasing interest in developing assay systems that are effective in detecting aneuploidy-producing agents. Because the current methodology for detecting aneuploidy is extremely varied, presently no comparisons of the validity and sensitivity of various assays can be made. This is due to a lack of sufficient data on the testing of the same compounds in multiple systems. Thus, there is an imminent need to select a few model compounds to be tested in all the available assays.

This chapter discusses the rationale for the selection of model compounds. Approximately 30 compounds were identified as candidate compounds for various reasons. It is not our intention to discourage studies of compounds not discussed in this chapter. It is merely our effort to facilitate the final selection of a few model compounds to be used for comparative studies in diverse assays or for collaborative studies to determine interlaboratory variation of selected assays.

INTRODUCTION

The ability of environmental agents to induce aneuploidy is of increasing concern, because aneuploidy induction in both meiotic and mitotic cells is significant in the development of genetic disorders, and may be for the development of cancers. In order to limit human exposure to potential aneuploidy-producing agents, it is necessary to establish effective and standardized assays with which to detect these agents.

The Aneuploidy Data Review Committee has studied literature on the induction of aneuploidy in 9 aneuploidy test systems by approximately 140 chemical compounds (57). While some of these compounds have been clearly shown to induce aneuploidy in some test systems, no chemical has been tested in all 9 test systems. Therefore, at the present time it is difficult to evaluate the validity and to compare the sensitivity of various aneuploidy test systems.

A general consensus from the Committee was the need to select a number of model compounds to be tested in available assays. Systematic comparisons of the performance of these compounds in various tests will enable the following questions to be addressed: (a) Can nonmammalian cells be effectively used to predict the aneuploidy-producing potential of compounds in mammalian cells? (b) Are compounds which cause aneuploidy in mitotic cells also likely to induce aneuploidy in meiotic cells and vice versa? (c) Do the in vitro test results correlate reasonably well with the in vivo results? In this chapter, a few candidate compounds will be suggested to be tested in each system.

Another area of concern of the Committee was the need to better understand the mechanisms of aneuploidy induction, because this will help in the development of improved assay systems. Thus, we will also discuss various potential targets for aneuploidy induction, and useful compounds which preferentially affect these targets.

CRITERIA FOR THE SELECTION OF MODEL COMPOUNDS

What types of compounds should be selected as model compounds? Although the opinions may vary among different investigators, we would like to suggest the following: (a) compounds that have been tested positive for aneuploidy induction in at least two different test systems; (b) compounds that are of environmental and clinical importance; and (c) compounds that have been shown to affect specific target organelles involved in cell division.

The first criterion is an easy one to justify because a model compound for the study of aneuploidy testing should have some evidence for its ability to produce aneuploidy. The second criterion represents compounds that may not have been widely tested, but have been shown or suspected to induce aneuploidy in limited studies. The rationale for choosing these compounds is that they are of environmental or clinical importance. These include some pesticides, industrial solvents and reagents, and various drugs. The third criterion is an important one, because if we understand the targets and the effects of compounds on these targets, then we will have a better opportunity to understand the mechanisms by which aneuploidy is induced. Naturally, the best choices of model compounds are those that fit into all three of these criteria. In this chapter, some candidate compounds that fit into each of these criteria and particularly those that fit into all three of these criteria will be identified.

COMPOUNDS THAT TESTED POSITIVE FOR ANEUPLOIDY INDUCTION IN MANY TEST SYSTEMS

From the literature review performed by the Aneuploidy Data Review Committee, 18 compounds which gave positive results in 2 or more different aneuploidy test systems were identified (57). A list of these compounds and their test results are shown in Tab. 1 and 2. Of these 18 compounds, 8 are known to specifically affect microtubules (MTs). They are benomyl, chloral hydrate, Colcemid, colchicine, griseofulvin, methyl-2-benzimidazole-carbamate (MBC), trifluralin, and vinblastine (3,11,17,23,48,60). Therefore, MTs seem to be an important target for aneuploidy induction. These compounds should be good candidates for model compounds. The second largest class of compounds are alkylating agents. They are cyclophosphamide, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), and methyl-N-nitronitrosoguanidine (MNNG). It is possible that they induce aneuploidy as a result of their mutagenic and clastogenic actions. For example, they may affect centromeric DNA sequences, and therefore, affect

Tab. 1. Compounds tested positive in at least 2 different aneuploidy test systems.

○ actinomycin D
atrazine
○● benomyl
○● chloral hydrate
○● Colcemid
● colchicine
○● cyclophosphamide
○ ethyl methanesulfonate (EMS)
○ p-fluorophenylalanine
● griseofulvin
○ methyl methanesulfonate (MMS)
○● N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)
● methotrexate
methyl 2-benzimidazole carbamate (MBC)
○ pyrimethamine (chloridin)
● 12-O-tetracadecannoyl-phorbol 13-acetate (TPA)
○ trifluralin
○● vinblastine

○ - Both mitotic and meiotic.
● - Both mammalian and nonmammalian.

chromosome segregation. But it is also possible that alkylation of nonDNA molecules, such as important division-related proteins and enzymes, may be primarily responsible for their aneuploidy-producing capabilities. Therefore, these compounds should be further investigated to elucidate the responsible targets. However, from the test development point of view, alkylating agents are not good candidate compounds because they can already be identified as hazardous compounds by a variety of mutagen assays. The purpose of developing new assays is to identify compounds that are harmful to human health, but might not be detected as posing a genetic hazard when only tested in standard assays for DNA damage.

Tab. 2. Performance of selected compounds in various aneuploidy test systems. From the literature review performed by the Aneuploidy Data Review Committee (57).

	Nonmammalian[a]					Mammalian[b]			
Compound	Mit ANN	Meio NCN	Mit/Meio SCN	Meio DMN	Mit/Meio PLN	Mit MCN	Mit MSN	Meio MFN	Meio MMN
actinomycin D	1+			2+					
atrazine		1+		1-	2+				
benomyl	4+	1-	1W	2-		1+,1-			
chloral hydrate	2+								1+
Colcemid				1+,1-		6+			
colchicine					4+,1-			1+	
cyclophosphamide	1+,1-	1-	1+					3+	1+
EMS			1+		1+				
p-fluorophenylalanine	2+	1+	1+						
griseofulvin	1+				1+	1+			
MMS	2+		1W						
MNNG	1W			1+,1-		1+			
methotrexate		1+						2+,1-	
MBC	1+		3+						
pyrimethamine							1+	1+	
TPA			1+			1+			
trifluralin	1W	1+							
vinblastine				1+,1-		2+			

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[a] ANN = Aspergillus; NCN = Neurospora; SCN = Saccharomyces; DMN = Drosophila; PLN = Higher plants; Mit = Mitotic; Meio = Meiotic; + = positive; - = negative; W = weak positive, # = Number of different studies.

[b] MCN = Mammalian cells in culture; MSN = Whole mammal somatic cells; MFN = Whole mammal female germ cells; MMN = Whole mammal male germ cells.

Of the 18 compounds shown in Tab. 1 and 2, 6 were found to induce aneuploidy in both mitotic and meiotic cells, and in either mammalian and nonmammalian cells. They are benomyl, chloral hydrate, Colcemid, cyclophosphamide, MNNG, and vinblastine. All of these agents, except cyclophosphamide and MNNG, should be choices for model compounds because they have been most widely studied, and their mechanisms of actions are known.

COMPOUNDS OF ENVIRONMENTAL AND CLINICAL IMPORTANCE

Table 3 lists a few examples of this class of compounds that have been either shown or suspected to induce aneuploidy. These compounds include some fungicides, herbicides, organic solvents, and clinically used anticancer, anesthetics, and other drugs. Of these, benomyl and chloral hydrate tested positive for aneuploidy induction in at least two different test systems. Therefore, they are the obvious choices. A few compounds, such as diethylstilbestrol (DES), vinblastine, taxol, diazepam, and griseofulvin, have been shown to induce mitotic arrest and aneuploidy in mammalian cell cultures (26,27,32,43,50,58). Therefore, they are also good candidates. Atrazine induces aneuploidy in fungi (21) and plants (34), however, its mechanisms of actions are not well understood. Trifluralin apparently affects MT assembly in plants but not in animal cells (23). It induces aneuploidy in fungi (12) but has not been evaluated for its ability to induce aneuploidy in animals or plants. Some organic solvents and anesthetics, such as the ones shown in Tab. 3, induce mitotic arrest and disrupt spindles, suggesting that they may be potentially aneuploidy-producing agents (36,51). However, they may not be suitable for testing in all systems because of their limited water solubility. Because the route of human exposure to these agents is via inhalation, relevant assays for testing these compounds are those in which cells can be exposed to the gaseous phase of these agents.

POTENTIAL TARGETS FOR ANEUPLOIDY INDUCTION

Before discussion of the selection of compounds that fit into the third criterion, it is necessary to review and speculate on the potential targets for aneuploidy induction. Since aneuploidy is induced as a result of chromosomal mis-segregation, structures that are thought to be involved in chromosomal segregation should be the major targets. These targets are shown in Tab. 4.

Tab. 3. Some examples of potentially aneuploidy-producing compounds that are of environmental and clinical importance.

Fungicides	Benomyl
Herbicides	Trifluralin, atrazine
Organic solvents	Benzene, chloroform, formaldehyde, trichloroethylene, and tetrachloroethylene
Anesthetics	Halothane, chloral hydrate
Anticancer drugs	Diethylstilbestrol, vinblastine, vincristine, taxol, and nocodazole
Antianxiety drugs	Diazepam
Antifungal drugs	Griseofulvin

Tab. 4. Potential targets for aneuploidy induction.

In Mitotic Cells	In Meiotic Cells
Spindle microtubules	Process of homologous pairing (e.g., formation of synaptonemal complex)
Centromeres/kinetochores	Process of crossing-over
Centrioles/centrosomes	
Microtubule-associated proteins and regulatory molecules	
Cytoplasmic membranes	
DNA	

In mitotic cells, the potential targets include the spindle MTs. Because many aneuploidy-producing agents are known to affect MTs, these are obvious targets. Another target is centromeres/kinetochores. Centromeres/kinetochores are regions on the chromosomes where spindle MTs are attached during chromosome movement to the poles. Inactivation or loss of kinetochores may result in chromosome lagging, and lead to aneuploidy. Premature separation or delayed separation of centromeres of sister chromatids may also conceivably cause aneuploidy. MT-associated proteins and regulatory molecules can be important targets, since MT assembly is influenced by many factors including the MT-associated proteins (MAPs), calcium regulatory proteins (e.g., Calmodulin), and various enzymes such as cAMP-dependent protein kinases and GTPase. Therefore, compounds that affect these proteins are likely to impair the function of the spindle, and lead to the induction of aneuploidy. Compounds that damage cell membranes can also conceivably produce aneuploidy by altering the membrane-tubulin association, or by impairing the function of the membrane in regulating the transport of important ions and enzymes that are necessary for cell division. The fact that several DNA-damaging agents have been shown to induce aneuploidy points to DNA as one possible target. It is not clear if all DNA or only specific DNA sequences such as the centromeric DNA are responsible for aneuploidy induction. Also, possibilities still exist that non-DNA molecules are the responsible targets for the aneuploidy-producing actions of mutagens and clastogens. Therefore, unlike mutagens and clastogens that have only one target, namely the DNA, aneuploidy-producing agents can have a variety of targets.

In meiotic cells, in addition to the above targets, processes that are unique in meiosis may also contribute to the induction of aneuploidy. These processes include the processes of chromosome pairing and crossing-over. Agents that interfere with the pairing or reduce the frequencies of crossing-over are also likely to induce aneuploidy. Furthermore, aneuploidy can result from mis-segregation of translocated chromosomes (e.g., ring IV) in first meiotic anaphase. Therefore, clastogens can produce aneuploidy in meiotic cells by this mechanism.

It should be noted that all of the above structures can be affected in interphase as well as mitosis. For example, spindle MTs may be affected as a result of damage occurring during S phase when tubulin messenger RNA is being transcribed, or G_2 phase when tubulin is synthesized. They may also be affected at prophase during astral and central spindle assembly, in prometaphase when MTs elongate, in anaphase A when kinetochore-to-pole MTs undergo disassembly, or in anaphase B when growth of interpolar MTs and interactions among MT fibers take place. Centrioles may be affected in various periods during their maturation (G_1 phase), replication (S phase), or separation (G_2 phase). Centromeres/kinetochores can also be affected during their replication (S phase), activation (prometaphase), and separation (metaphase and anaphase).

Because the cell division processes are extremely complex and dynamic, there may be other potential targets that are not mentioned above. The targets discussed above only represent those that are known to be affected by some chemical compounds. Studies of the aneuploidy-producing potentials of these compounds should lead to a better understanding of the mechanisms of aneuploidy induction.

COMPOUNDS THAT HAVE BEEN SHOWN TO AFFECT SPECIFIC TARGET ORGANELLES

Compounds that Interfere with Microtubule Assembly

A long list of compounds have been shown to affect MT assembly (15). Only a limited number of these agents will be discussed here (Tab. 5). Because of the different actions exerted by various compounds on the MT, selected agents with different actions will be discussed separately.

Agents that inhibit polymerization of tubulins. Colchicine, Colcemid, and podophyllotoxin are examples of these agents. These 3 compounds appear to share a similar binding site on the tubulin (11,31). All of them cause mitotic arrest. Benzimidazole derivatives such as benomyl, methyl-2-benzimidazole-carbamate (MBC), and nocodazole also inhibit polymerization of tubulins and induce mitotic arrest (17,25,61). Nocodazole appears to bind MTs at the same site as colchicine (24). But contrary to colchicine, which has no action on the MTs of the fungi *Physarum polycephalum*, nocodazole is a strong inhibitor of their tubulin assembly and cell growth (47). The only other mitotic poison effective in this species is griseofulvin (22). Therefore, by comparing the actions of nocodazole or griseofulvin with those of colchicine in lower eukaryotic and mammalian cells, one could gain some insights into the differences in their aneuploidy-producing capabilities in these two types of cells.

Agents that bind to the sulfhydryl residues of protein molecules have also been shown to prevent tubulin polymerization. For example, diamide, a sulfhydryl-oxidizing agent, inhibits the polymerization of tubulin, and its effect can be reversed by dithioerythreitol, a sulfhydryl-reducing agent

Tab. 5. Examples of compounds that have been shown to affect microtubules.

Agents that affect polymerization of tubulin

colchicine
Colcemid
podophyllotoxin
benzimidazole derivatives (benomyl, MBC, nocodazole)
griseofulvin
sulfhydryl reagents (mercury compounds, arsenic compounds, diamide)
diethylstilbestrol
para-fluorophenylalanine

Agents that crystallize tubulin

Vinca alkaloids (vinblastine, vincristine)

Agents that enhance polymerization of tubulin

Taxol

Agents that affect anaphase spindle movement

Taxol inhibits chromosome-to-pole movement.
Chloral hydrate blocks pole-to-pole spindle elongation.

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(40). Further studies indicated that two sulfhydryl residues close to the colchicine binding site on the tubulin dimers are essential for tubulin polymerization (33,55). Organic mercury compounds also bind to sulfhydryl residues on tubulins and prevent polymerization (28). These compounds also induce mitotic arrest in cultured mammalian cells (41) and aneuploidy in oocytes of Syrian hamsters (39).

Other compounds of interest are DES and p-fluorophenylalanine (pFPA). DES induces mitotic arrest and aneuploidy in mammalian cell cultures (52, 58). It affects MT orientation, kinetochore and centriole interactions, and centriole structure (Brinkley et al., this Volume). pFPA is presumably incorporated into division-related proteins (56) and has been shown to have an effect on tubulin in *Aspergillus* (42). It is a potent inducer of aneuploidy in lower eukaryotes (20).

Agents that crystallize tubulin. Vinca alkaloids such as vinblastine and vincristine have a characteristic action on the tubulin of mammalian cells, i.e., the assembly of tubulin into crystalline structures (3). They bind to tubulin at two different sites. One is similar to the colchicine-binding site which prevents tubulin assembly, and the other is a unique site which causes lateral aggregation of tubulins. Both of these agents are used clinically as antineoplastic agents. They both arrest and accumulate metaphases in mammalian cell cultures (27). Vinblastine has been shown to induce aneuploidy in both cultured Chinese hamster cells (28) and mouse bone marrow cells in vivo (35).

Agents that enhance polymerization of tubulin. Taxol is a unique compound in that it lowers the critical concentrations of tubulins required for assembly (13). As a result, it favors the formation of MTs (53) and increases the stability of MTs (54). The failure of a cell to disassemble its MTs and form a mitotic spindle results in mitotic arrest (18). Taxol has also been shown to induce aneuploidy in mammalian cell cultures (50). This compound is increasingly being used in microtubule research to study the structure and function of MTs.

Agents that affect anaphase spindles. After the metaphase spindle is formed, the movement of the spindle during anaphase may be affected by chemical agents. There are two types of anaphase spindles: interpolar MTs and kinetochore-to-pole MTs. Anaphase is characterized by two processes: shortening of the kinetochore-to-pole MTs followed by elongation of the pole-to-pole MTs. The former occurs at anaphase A, and the latter at anaphase B. Taxol is a compound that specifically inhibits the movement of the kinetochore-to-pole MTs but does not affect spindle elongation (8). On the contrary, chloral hydrate blocks spindle elongation but does not affect kinetochore-to-pole movement (48). Therefore, comparisons of the aneuploidy-producing abilities of these two agents should yield information on some aspects of the processes of chromosomal segregation.

Thus, a large number of chemical compounds are known to interfere with MTs by different mechanisms (Tab. 6). These compounds should be useful for studying the involvement of MTs in inducing aneuploidy in various organisms and cell types.

Compounds that Affect Centromeres/Kinetochores

Few compounds are known to affect centromeres/kinetochores. Colchicine was shown to alter the trilamellar structure of kinetochores (6,49). However, it apparently does not affect the binding of spindle MTs to kinetochores. Mitomycin C was shown to remove kinetochores from chromosomes (7). This effect is likely to be the result of delayed separation of centromeres of sister chromatids (16). Thus, this compound has the potential

Tab. 6. Examples of compounds that have been shown to affect targets other than microtubules.

Compounds that affect centrioles/centrosomes

diazepam
diethylstilbestrol
ethidium bromide
actinomycin D

Compounds that affect centromeres/kinetochores

colchicine
mitomycin C

Compounds that affect microtubule-associated proteins and regulatory molecules

chlorpromazine
W-13

Compounds that affect cell membrane

amphotericin B
fenarimol
miconazole

Compounds that affect chromosome pairing and crossing-over

colchicine
cytosine arabinoside

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to induce aneuploidy by damaging the kinetochores. Premature separation of centromeres has been proposed as one mechanism for the induction of nondisjunction (59). However, compounds that induce aneuploidy by such a mechanism are yet to be identified.

Compounds that Affect Centrioles/Centrosomes

Centrioles and associated pericentriolar materials (centrosomes) serve as spindle poles toward which chromosomes are moved during cell divisions. Compounds that damage centrioles/centrosomes may cause multipolar formation or lagging chromosomes, and lead to aneuploidy induction. Since many eukaryotic cells lack centrioles (e.g., fungi, plants, and mouse oocytes), these structures do not appear to be essential for cell division. However, the compound diazepam, which inhibits the separation of centrioles (1), has been shown to induce aneuploidy in cultured Chinese hamster cells (26). Further studies are needed to determine the molecular mechanisms for diazepam-induced aneuploidy. By testing this compound on cells lacking the centrioles, one would gain some insights into the roles of centrioles in cell division. Another aneuploidy-producing agent that affects centrioles is DES. It causes elongation of centrioles (Brinkley et al., this Volume). This compound apparently also affects MTs. Therefore, the mechanisms of aneuploidy induction by DES may be different than those by diazepam. Ethidium bromide damages centrioles by yet another mechanism (38). It causes structural damages of centrioles (e.g., broken rings) in mammalian cell cultures; it also induces chromosomal endoreduplication and polyploidy (37). Possible induction of aneuploidy by this compound has not yet been examined. Actinomycin D inhibits centriole duplication in mammalian cells by inhibiting RNA synthesis (14). It induces aneuploidy in both *Drosophila* and *Aspergillus*. However, its ability to induce aneuploidy in mammalian cells has not been examined.

Compounds that Affect Microtubule-associated Proteins and Regulatory Molecules

Microtubule assembly is influenced by many factors including calmodulins (Ca^{2+}-regulatory proteins), MT-associated proteins (e.g., MAPs, Tau, HMW proteins), and various enzymes (e.g., cAMP-dependent protein kinase, GTPase) (10,15). While the exact roles of these factors in MT assembly and disassembly are not well understood, inhibitors of these factors may be used to reveal the functions of these proteins. The compound, chlorpromazine, has been shown to bind calmodulin (29). However, this binding is nonspecific because it also binds to actin and tubulin. Nevertheless, it has been shown to induce metaphase arrest and accumulation in cultured Chinese hamster cells (5). Because most mitotic arrestants have been shown to induce aneuploidy, chlorpromazine should be a potential aneuploidy-producing agent. The compound W13 has a more specific binding to calmodulin than chlorpromazine (9). It is an extremely useful compound because its analog, W12, does not bind to calmodulin. Therefore, a comparative study on their aneuploidy-producing potentials can be made. However, the availability of this compound has been a problem.

Compounds that Affect Cell Membranes

Compounds that damage cell membranes may cause aneuploidy by either altering tubulin-membrane associations or impairing the regulations of the transport of ions, transmembrane signals (i.e., adenyl cyclase), and enzymes that are necessary for normal cell division. It has been clearly demonstrated in algae that MTs and membranes form close associations at some parts of the cell membrane (44). The involvement of cell membranes in chromosomal segregation has been demonstrated in fungi by Bellincampi et al. (2). They have shown that a membrane-damaging compound, amphotericin B, disturbs normal cell division by altering the distribution of chromosomes and leads to mitotic nondisjunction. Although the mitotic apparatus in mammalian cells appears to be isolated from the cell membrane, the possibilities of membrane involvement in chromosomal segregation can not be excluded. In fact, there are some indications that membrane-bound tubulins are present in mammalian cells (4). Whether or not these membrane-bound tubulins are involved in chromosomal segregation remains to be seen. Amphotericin B should be a good model compound with which to probe the role of mammalian cell membranes in chromosomal segregation. Other membrane-damaging compounds, such as fenarimol, which interferes with ergosterol synthesis, and miconazole, which affects membrane structure and permeability, can also be good candidates (2). These fungicides have been shown to induce aneuploidy in fungi (2).

Compounds that Affect Chromosomal Pairing and Crossing-over in Meiosis

Chromosomal pairing and crossing-over are mechanisms to ensure proper segregations of homologs during first meiotic divisions. Agents that interfere with normal processes of chromosomal synapsis and recombination are likely to induce aneuploidy. At an elevated temperature, reduction of the frequencies of crossing-over has been correlated with aneuploidy induction in *Drosophila* (19). Cold treatment has also been shown to prevent chromosomal pairing leading to aneuploidy induction in mouse oocytes (30). Few chemical compounds have been examined for their effects on meiotic pairing. Colchicine, when applied to premeiotic stages in plants, was found to reduce chiasma frequencies and increase the frequencies of univalents at metaphase I (46). Cytosine arabinoside was shown to interfere with meiotic pairing in mouse spermatocytes (45). These two compounds may be candidates for future studies of the relationship between meiotic pairing and aneuploidy induction.

SUMMARY OF THE SELECTION OF MODEL COMPOUNDS

Table 7 shows the summary of selected compounds that fit into at least one of the three criteria discussed above for model compounds. Of the 31 compounds, 10 appeared to be the best candidates because they are likely to fit into all 3 criteria. These are benomyl, chloral hydrate, colchicine, diazepam, DES, griseofulvin, mercuric chloride, taxol, trifluralin, and vinblastine. Since most of these compounds affect MTs, it is necessary to choose some model compounds that affect targets other than MTs. For example, mitomycin C which affects kinetochores; ethidium bromide which causes structural damages of centrioles; amphotericin B, fenarimol, and miconazole which damage cell membranes; and cytosine arabinoside which affects meiotic pairing.

Tab. 7. Summary of selected compounds that fit into at least one of the criteria for model compounds.

Compounds[a]	Tested positive[b]	Environmental importance	Specific targets[c]
actinomycin D	(+)	+	(+)
amphotericin B*	(+)		+
atrazine	+	+	
benomyl**	+	+	+
benzene		+	
chloral hydrate**	+	+	+
chloroform		+	
chlorpromazine		+	(+)
Colcemid	+		+
colchicine**	+	+	+
cyclophosphamide	+	+	(+)
cytosine arabinoside*		+	(+)
diazepam**	(+)m	+	+
diethylstilbestrol**	(+)m	+	+
ethidium bromide*			+
fenarimol*	(+)	+	+
p-fluorophenylalanine	+		(+)
formaldehyde		+	
griseofulvin**	+	+	+
halothane		+	+
mitomycin C*		+	+
mercuric chloride**	(+)m	+	+
miconazole*	(+)	+	+
MNNG	+		(+)
nocodazole		+	+
taxol**	(+)m	+	+
tetrachloroethylene		+	
trichloroethylene		+	
trifluralin**	+	+	(+)
vinblastine**	+	+	+
vincristine		+	+

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[a] Compounds with ** are the best candidates for model compounds because they induce aneuploidy in mammalian cells, are widely used in the environment, and affect specific targets. Compounds with * are considered good candidates for studies of the involvement of different target organelles in aneuploidy induction in different cell types.

[b] + indicates positive for aneuploidy induction in at least 2 different assays. (+) indicates positive in only one assay. (+)m indicates positive in a mammalian test system.

[c] + indicates compounds with specific targets on microtubules, centromeres/kinetochores, centrioles/centrosomes, calmodulin, cell membrane, or meiotic pairing. (+) indicates compounds that affect the above targets less specifically or in a less known manner than those indicated as +.

Studies of various compounds with different actions will not only allow us to better understand the mechanisms of aneuploidy induction, but also will help in the development of new assays that are more effective than the existing ones in detecting aneuploidy-producing agents.

CONCLUSION

Unlike mutagenic or clastogenic compounds whose principal target is DNA, agents that induce aneuploidy may not act on DNA, but rather on a variety of components or structures responsible for cell division. Consequently, studies on the mechanisms of aneuploidy induction are much more complex than those of gene mutations or chromosome aberrations. This chapter has reviewed some of the potential targets for aneuploidy-inducing agents, and has discussed the selection of a number of model compounds with which to probe the processes that lead to chromosomal nondisjunction. It is important to remember that chemical compounds may produce a number of different cytological effects unrelated to their aneuploidy-producing capabilities. Not until a sufficient number of compounds with different actions is tested in various test systems can we begin to understand the mechanisms of aneuploidy induction in different organisms and cells.

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DISCUSSION

ABBONDANDOLO: I would like to make a remark on the aspect of the possible cellular targets of chemicals causing aneuploidy. We have shown in a poster presented here that there is the possibility that alkylating agents, which of course are agents that damage DNA, might also have an unsuspected, different cellular target, from the point of view of induction of aneuploidy. Our hypothesis is that if the akylating agents also alkylate nucleotides, which are present in the cellular precursor pool (as, in fact, they do), and if we assume that some of these alkylated nucleotides (and particularly, the alkylated guanine nucleotides) can interfere with the formation of the spindle fibers, then perhaps we might have an unsuspected, different target for this well known class of chemical agents. So as Dr. Liang has stressed, it may be the case that for some class of chemical agents, we have a pleiotropic effect with more than one cellular target involved.

BRINKLEY: That is a very good point. It falls under the category of regulation which we have not discussed at length during this Symposium.

BOOTHROYD: I have a comment that applies very much to centrioles. We all know that there are many systems, even in the mammalian system (e.g., oocytes), that do not have them. I think that we should be very cautious in including the centriole as a prime target.

BRINKLEY: Centrioles are just markers to show us where these important microtubule organizing centers (MTOCs) are located in the cell. It happens that centrioles are synchronized in their replication with the cell cycle. They not only tell us where the MTOCs are, they even indicate cell-cycle stage. For example, if you see a single pair, you know the organizing center is sitting in a cell in G_1. If the centriole is doubled, the cell is in G_2, about to go into mitosis. So they are useful markers, but I think your point is well taken. Centrioles per se may not regulate, or have anything to do with the events that cause aneuploidy. However, it is very interesting that drugs that affect centrioles do affect the incidence of aneuploidy.

BOOTHROYD: I think we might perhaps consider the centriole as a passenger rather than as the organizing center.

BRINKLEY: That is right. I believe that you are very correct in that it is a passenger.

SHARMA: I think it is important in testing to detect the positive compounds as positives. But at the same time it is important that compounds that are negative are also detected as negative in the same test systems. So don't you think it would be appropriate to include one or two chemicals which are negative for aneuploidy induction, along with the chemicals which are positive that you have listed?

LIANG: Positive compounds are important in test development and, of course, negative compounds should be included in test validation.

GAULDEN: I think there is one area with respect to chemical agents that we really have not given much attention to, and that is cell surface receptors.

BRINKLEY: We are back to regulation again. That whole zone of something in the extracellular system, hormone or whatever, that stimulates cells to go into division, and the whole series of events from the receptor to the initiation of a cell to go into mitosis, have not been covered here because we do not know much about these processes. But regulation is extremely important.

CARERE: I have two comments. One on the membrane as a target; one on the findings from Abbondandolo. Amphotercin B acts by complexing steroids in membranes. This has been found in Aspergillus by Gualandi of our group. Is it possible to consider another chemical which acts specifically on some steps in the metabolism of steroids? Finally, regarding the findings from Abbondandolo. We usually try to distinguish in a very simplistic way between genotoxic agents acting on DNA, including alkylating agents, and other chemicals like benomyl and such. Alkylating agents can induce aneuploidy through more than one mechanism. This means that it is possible that they can also act on DNA and on the pool of the nucleotides as Abbondandolo has found.

MECHANISMS TO STIMULATE RESEARCH ON ASSAY SYSTEMS TO DETECT ANEUPLOIDY

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INTRODUCTION

It is clear that aneuploidy presents a serious human health hazard and that the incidences of Down syndrome, Turner syndrome, Klinefelter syndrome, etc., are not insignificant in the human population (Hook; Evans, this Volume). It is also clear that to provide adequate medical care for such individuals involves substantial expenditure of funds at both the State and Federal levels (Edel and Edel, this Volume). The fact that aneuploidy can be produced by both physical and chemical agents raises the concern in many government agencies that there may be untested chemicals in the environment with the potential to cause increased frequencies of aneuploidy in the human population. There is an obvious need to screen environmental chemicals to identity potential chemicals inducing aneuploidy in experimental assay systems. But what chemicals could be used as positive controls and which assays should be used to detect those chemicals that would be active in the human population are both unresolved questions. The literature survey of chemicals that induce aneuploidy (Dellarco et al.; Waters et al., this Volume) has shown that the database is very limited; there are only about 20 chemicals that have been tested in 3 or more assays. We have the beginnings of a list of chemicals that are known to induce aneuploidy, but only limited information on the range of organisms affected by a particular chemical. In general, we do not have sufficient information to know whether or not a chemical that produces aneuploidy in yeast will also produce aneuploidy in the whole animal (including man). Alternatively, there is insufficient information to know whether chemicals that are active in mammals are also active in those lower eukaryotes that could be used in mass-screening programs.

MECHANISMS TO STIMULATE METHOD DEVELOPMENT

One simple mechanism to stimulate research on the development of new assays to test chemicals for the induction of aneuploidy would be to establish chemical and data repositories. It would be a simple matter for the Environmental Mutagen Information Center to provide a periodic evaluation of published data and to add to the recent literature reviews (Dellarco et al.; Waters et al., this Volume). The concept of a chemical repository is new, but may have merit for a variety of reasons. The repository should contain those chemicals that have been shown to be active in various assay systems; new chemicals would be added as the database grows. Because chem-

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icals from different sources can vary in activity as a result of varying amounts of contaminants, the repository should contain a reference sample, which has demonstrated activity, that would be available upon request to other investigators. Chemicals stored in the repository should be "pure" compounds rather than technical grade reagents. This might require some retesting when a chemical selected for storage in the repository turns out to be a technical grade rather than a highly purified sample. The chapters in this Volume show that there are a variety of mechanisms by which aneuploidy can be induced, and chemicals have already been found with highly specific activity (Liang and Brinkley, this Volume). This repository could serve as a source of reference chemicals to which the activity of other chemicals could be compared. Chemicals selected as reference chemicals would have known levels of activity in particular assay systems and would provide a basis for comparison (as positive controls) when tests were made on new chemicals and when new assay systems were developed. An additional justification for establishing a repository would be that many laboratories do not have the resources required to evaluate the purity of chemicals obtained from different chemical supply companies and the problems associated with impure or technical grade reagents could be avoided by investigators starting research programs to develop new tests to detect aneuploidy.

MASS-SCREENING PROGRAMS TO DETECT ANEUPLOIDY-INDUCING CHEMICALS IN THE ENVIRONMENT

It is well-established that there is a need to screen chemicals in the environment to which the human population is exposed, to determine whether such chemicals can induce aneuploidy. The need was apparent at least 10 years ago and was actually the basis for the workshop that was organized by the National Institute of Environmental Health Sciences (NIEHS) in November, 1978 (Environmental Health Perspectives, Vol. 31, August, 1979). It was in response to that perceived need that the National Toxicology Program developed collaborative research projects (Mason and Resnick, this Volume), both with Drosophila and yeast. The desired outcome of this work will be assay systems that can be used in mass-screening programs.

The problem that we have is that because the database is so limited (Dellarco et al.; Waters et al., this Volume), we do not know whether these assays will provide a comprehensive screen that will detect all chemicals with the potential to cause aneuploidy in man. At the beginning of such a test program, the chemicals that are known to be active in yeast and/or Drosophila will have to be subjected to further tests in higher eukaryote assay systems as they are developed and validated. At this point in development, there are two alternative strategies for database development and expansion. The establishment of a repository will encourage the testing of chemicals with demonstrated activity in other assay systems, both those already in use as well as those that will be developed in recognition of needs discussed in this Volume.

An alternative approach would be the development of a collaborative trial in which a specific set of chemicals could be tested in a wide range of assay systems. This latter approach has the potential to develop an expanded database to provide the desired information in 2 or 3 years. The first approach would require considerably more time and provide an extremely limited database as the recent literature surveys have adequately demonstrated (Dellarco et al.; Waters et al., this Volume). International Collaborative Trials have been used quite successfully over the past 8 years to evaluate short-term tests for carcinogens (1,2). This same mechanism could be used to evaluate those assay systems that can detect aneuploidy. This type of program not only would make it possible to evaluate the efficacy of a given assay, but could also determine whether comparable data could be obtained with the same assay system in different laboratories. With the appropriate design, the study could determine the range of activity of chemicals which have been shown to be active in a limited number of assay systems.

International Collaborative Trials to Evaluate Short-term Tests for Carcinogens

In the first trial which was started in 1977, the International Program to Evaluate Short-term Tests for Carcinogens (IPESTTC), a total of 42 chemicals were used to evaluate the utility of 36 different assay systems (2). The 42 chemicals included 25 known carcinogens as well as 17 noncarcinogens. This study was followed by another International Collaborative Study that was started in 1981 sponsored by the International Programme on Chemical Safety (IPCS), a joint program of the World Health Organization, the International Labor Organization, and the United Nations Environmental Program. The first IPCS Trial was designed to evaluate the utility of eukaryotic assays to detect those chemical carcinogens that could not be detected, or detected only with difficulty, with the Ames Salmonella test (1). A total of 10 chemicals were tested in a total of 47 assay systems ranging from fungi to mammalian cells in culture. The second IPCS International Collaborative Study, which was started in 1983, was designed to evaluate short-term in vivo assays (Ashby et al., in prep.). Two carcinogen--noncarcinogen pairs of chemicals are being tested in 21 major groups of assays. All 4 chemicals are positive in vitro, but new data from selected in vivo test systems are showing negative tests for the two noncarcinogens. Thus the new data on these 4 chemicals are in better agreement with the Rodent Bioassay Data than the original data from the in vitro assays. The selective utilization of specific in vivo assays may provide a useful mechanism to eliminate any false positives arising from the use of in vitro assays in mass-screening programs.

Selection of chemicals. The selection of chemicals for a collaborative trial on assay systems to detect aneuploidy depends on a number of factors. Primary consideration should be given to those chemicals with demonstrated activity in both lower and higher eukaryotic organisms. This selection is based on the rationale that lower eukaryotes will provide a mechanism for rapid and inexpensive screening of chemicals for the potential to produce aneuploidy in higher eukaryotes, including man. Special care will have to be taken to determine whether chemicals produce the same effects in mitosis and meiosis and whether similar effects are found in both higher and lower eukaryotic organisms. If there is more than one mechanism for inducing aneuploidy, then chemicals that preferentially interfere with a particular mechanism will also make suitable candidate chemicals even if the database is somewhat limited (Liang and Brinkley, this Volume).

Other questions concerning chemical selection relate to the number of chemicals to be tested. Experience from other studies indicates a maximum of 10 to 20. If whole animal assays are to be evaluated, many investigators will find testing 10 chemicals a major effort. The coding of chemicals needs also to be discussed: should the chemicals be tested as coded samples with decoding only after final data have been submitted? Does coding of known samples of chemicals serve any useful purpose in such a collaborative trial? All chemicals should be readily soluble in water, preferably solids, and stable with regard both to light and ambient temperature. Each of the chemicals will be prepared in a single lot and subdivided so that aliquots can be distributed to all investigators. This latter approach will minimize interlaboratory variation that might result from the use of samples with varying levels of contamination that result from the purchase of these samples from different commercial sources. It will also be essential for the investigators using the same assay to develop a standardized protocol so that all of the data derived from the use of that assay system can be directly compared. This approach will make it possible to develop a database to evaluate interlaboratory variations and the reproducibility of data on each of the test chemicals in geographically isolated locations.

Investigator certification. Some mechanism needs to be established to determine whether the investigators proposed for participation in such a collaborative trial have sufficient expertise to provide a comprehensive evaluation of the proposed test chemicals in a particular assay system. The proposed study should not be used as a training exercise. Ideally, the investigators will have had considerable experience with the use of a particular assay and their function will be to compare the effects of the test chemicals with appropriate positive and negative controls.

SUMMARY

The present database on the induction of aneuploidy is inadequate to make comparisons between effects on lower eukaryotic and higher eukaryotic organisms. There is also an urgent need to develop new assays to detect the induction of aneuploidy in animals, as well as assays in lower eukaryotic organisms which can be used in mass-screening programs. The development of chemical and data repositories of chemicals that induce aneuploidy with known activity is proposed as a mechanism to stimulate research in this area.

An efficient mechanism to develop a comprehensive database on particular assay systems and a defined set of chemicals is an International Collaborative Trial developed under and sponsored by the International Program on Chemical Safety at the World Health Organization. IPCS is sponsored by voluntary contributions from those member countries who consider such collaborative studies of importance for the development of methodology that can be used to evaluate the mutagenic and carcinogenic potential of environmental chemicals. Exposure to natural and manmade chemicals can be unique, and member countries cannot necessarily assume that chemicals to which native populations will be exposed will be tested elsewhere.

The development of an International Collaborative Trial on chemicals that induce aneuploidy and a wide range of eukaryotic assay systems provide an excellent mechanism for the development of a comprehensive database. Within 2-3 years these new data could help to resolve some of the numerous questions that have arisen during the course of the research presented in this Volume.

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DISCUSSION

BECKING: I am with the World Health Organization International Program on Chemical Safety and I would like to amplify and clarify some of the things that have been said. In Geneva there is a group that has the prime responsibility for methods development. But that is actually only one goal out of six for the program. Collaborative studies are one small part of that goal on methods development. They are very costly, both in time and money, both for the Central Unit in Geneva, as well as for the Institutes. Dr. de Serres can verify that. Because of his diligence, we did succeed in two collaborative studies in the area of genotoxicity. We did benefit from a database on carcinogenicity in that study, which I do not think exists for aneuploidy. We have human carcinogens, or we have animal carcinogens, to which you can then evaluate your test systems. The point I am trying to make is that given the potential health risk from aneuploidy in man, it is a program which the IPCS really wants to take a long look at. We need the advice of people that are at this Symposium. If any want to write me about the possibilities of collaborative studies, I can use all the help I can get. We need to look at it, but we need to look at it when the science dictates an international collaborative study, and not before.

FUTURE DIRECTIONS IN TEST SYSTEM DEVELOPMENT AND VALIDATION

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INTRODUCTION

It is widely recognized that the frequency of aneuploidy in man is very high. An estimated 0.3% of livebirths are aneuploid and this figure represents the legacy of an even higher incidence of aneuploidy at conception (18). Since there is also incontrovertible evidence that the frequency of aneuploidy can be increased in experimental organisms by a variety of treatments, there is naturally a concern that human populations, if exposed to aneuploid inducers, might suffer increased aneuploidy. The question which then arises is how best to monitor the environment for such inducers.

There are difficulties in designing a screening program which arise from features peculiar to aneuploid induction, as follows:

(a) Spontaneous aneuploidy can arise from a variety of defects (see Ref. 6 for review). It is important to realize that we are almost totally ignorant of the relative importance of these defects in generating aneuploidy. Thus Hassold and Jacobs (18) wrote, "... virtually nothing is known about the mechanisms leading to trisomy in man ... we do not even know whether the primary event in trisomy involves the chromosome itself, the spindle, or some other cellular organelle."

(b) Inducers of aneuploidy may affect some of these processes and not others. Ford's classification (12) of the causes of aneuploidy is useful here. He recognized immediate causes related to the "properties of the chromosomes themselves ... and processes within the cell external to the chromosomes," and secondary causes which include physiological processes, such as aging, that modify aneuploidy frequency. Aneuploidy inducers may act on either the immediate or secondary causes, and this diversity of possible action increases the difficulty of identifying them experimentally.

(c) When aneuploid frequency is increased following an experimental treatment the increases are characteristically quite small. Although there are some exceptions--for example, colchicine treatment of female Chinese hamsters increased aneuploidy from 2% to 25.9% (34)--most treatments seem to induce much smaller increases

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(perhaps doubling the spontaneous frequency). This obviously increases the cost of obtaining statistically significant data and thus increases the difficulties of identifying aneuploid inducers.

(d) Although, broadly speaking, cell division is an evolutionarily conserved process, its details do vary between organisms and this leads to real problems in extrapolating any findings from experimental organisms to man. These difficulties may arise because the direct action of a treatment is peculiar to the test organism or because a treatment has an effect via secondary physiological processes which are confined to the test system. Identified examples of the first type are compounds acting directly on the cell division spindle. It is firmly established that the spindle of lower eukaryotes has a different sensitivity to some tubulin-binding compounds compared to higher eukaryotes (8,19). There are no confirmed examples of induction via secondary causes, but an obvious possibility would be any treatment which enhances physiological age. This might result in increased aneuploidy, but such an effect would be confined to organisms with an identifiable age effect.

Any screening strategy which is adopted to try and identify any environmental hazard must be structured so that it takes these problems into account. It is also important to bear in mind the questions to which answers are required if the program is to be successful. To my mind some of these important questions are:

(a) How reliably can results with experimental organisms be extrapolated to man? In particular, what sort of indicator are results obtained from lower eukaryote test systems?

(b) To what extent do aneuploidy tests identify an unique environmental danger? It is already known that some substances increase aneuploidy as their sole genetic endpoint, that is they are not mutagenic in standard mutation tests. Whether such substances constitute an unique class targeting onto one particular part of the cell or whether there are many potential targets is not known.

(c) Is it generally true that induction of aneuploidy is proportional to the spontaneous level of aneuploidy in that organism? There seems to be no obvious mechanistic reason why organisms with a high spontaneous level of aneuploidy should also exhibit increased sensitivity to inducers, but nonetheless in several experimental systems this seems to be the case. Thus, in *Sordaria brevicollis* strains carrying a chromosome translocation which increases spontaneous aneuploidy about a hundred-fold, and in chromosomally normal strains, both show a 40% increase over their characteristic spontaneous rate when treated with a 70 mM solution of dimethylsulfoxide (15). A similar finding has been made in yeast when strains with a range of spontaneous frequencies have been exposed to inducers (S.G. Whittaker, pers. comm.). Just how generally this observation holds in other organisms remains to be established. It could be tested relatively easily in organisms like Drosophila or the mouse where stocks with different spontaneous frequencies are available. The answer to this question is of considerable importance because if it is generally true it will mean that man might be especially vulnerable to inducers.

These questions are of a general nature. If a coordinated program of research activity can provide answers to them we will have gone a long way, not only towards developing an aneuploidy screening method, but also towards understanding the underlying mechanisms of aneuploid production. There are also more specific questions which arise when the available testing systems and the selection of chemicals to be tested are considered.

METHODS OF DETECTING ANEUPLOID INDUCTION

It is not my intention to review exhaustively all the available methods. Rather, I wish to draw attention to strengths and weaknesses of particular systems because these are the primary constraints on the design of any screening program.

One problem connected with the available test methods is of overwhelming importance. The experimentally convenient microbial systems are taxonomically remote from man and, as mentioned above, are known to have spindles with different sensitivity to some inhibitors. In addition the physiology of these organisms is so different that chemical compounds inducing aneuploidy in man through secondary events might well go undetected if only microbial tests were to be relied on. Mammalian test systems, on the other hand, all suffer from the fact that they are time-consuming, labor-intensive, and therefore expensive.

Microbial Test System

There are several different types of microbial systems all of which detect aneuploidy genetically, inferring the presence or absence of a chromosome through anomalous genetic segregation. Multiply-marked chromosomes (16,26,28,32), allelic complementation (14), and gene dosage (36) have all been used to detect aneuploidy. Of these methods the latter has the considerable advantage that all cells which are either disomic or trisomic for the marked chromosome are detected. This yeast system would appear to have the biggest advantages. It is experimentally convenient and inexpensive and has been shown to be capable of detecting induction. There is, however, one feature of yeast biology which has to be borne in mind when considering induction. When dividing, each yeast chromosome is thought to be attached to the spindle by a single microtubule (5), which might make yeast cells unusually susceptible to aneuploid induction by spindle poisons. The results using yeast which have been presented in this Volume illustrate this point. Large increases in aneuploidy following treatment have been detected using a variety of substances, and these results are in sharp contrast with the smaller increases which appear to be characteristic of induction in, for example, Drosophila. These large increases could make yeast an ideal system, but, alternatively, tests might generate a large number of positive results which are of little relevance as an indicator of potential danger to a mammalian cell.

Aspergillus nidulans has also been widely used to monitor aneuploid inducers (4,11,21,26). Many of the substances shown to induce aneuploidy in this system do not induce gene mutations, which indicates that a target other than DNA is involved. Cyclophosphamide treatment was shown by De Bertoldi et al. (11) to increase aneuploidy in this fungus, but only after activation with S9 microsomal mix. The main disadvantage of Aspergillus systems is that they monitor mitotic nondisjunction only.

The system developed in Sordaria brevicollis detects aneuploidy of meiotic origin and has the advantage that several different meiotic defects can be distinguished (14). It has the disadvantage, however, that it is the most labor-intensive of the microbial systems, although a recent modification to the testing protocol has reduced the labor involved in testing a given compound (15).

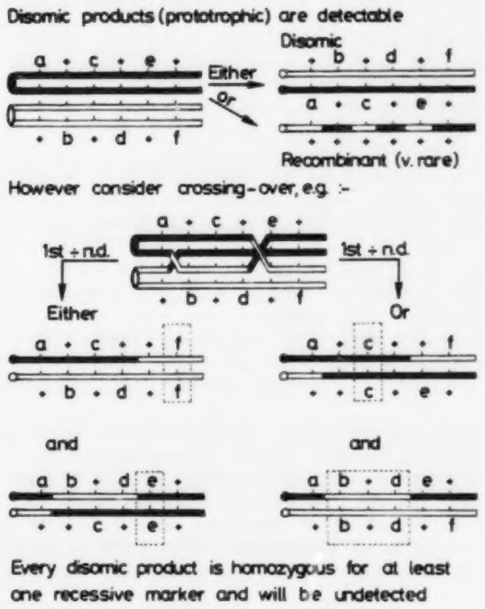
It is important to realize the strengths and weaknesses of particular systems and to design the validation program so that a range of test methods are appraised. For example, some systems use multiply-marked chromosomes and rely on a staggered arrangement of the markers so that a prototrophic phenotype is most likely to arise from nondisjunction rather than recombination. Fig. 1 illustrates how such methods are biased in favor of detecting compounds which interfere with recombination frequency. Any treatment which lowers recombination in the marked chromosome will result in an apparent increase in aneuploid frequency as the proportion of aneuploid products which will be undetected decreases.

Nonmicrobial Test Systems

In higher organisms both genetic and cytological methods are available. While genetic methods are nearly all confined to detecting aneuploidy among liveborns, cytological methods can analyze aneuploidy at many different stages of development.

Genetic Methods

In Drosophila, detection of sex chromosome aneuploidy is the simplest and best studied method. The aneuploids are viable and recognized by the exceptional inheritance of genetic markers on the sex chromosomes. For autosomal aneuploidy the problem to be overcome is that the genetic imbalance associated with the aneuploidy is incompatible with a viable existence. In the isochromosome method as used by Bateman (3) treated animals are crossed to tester strains which carry a chromosome rearrangement. Any


Disomic products (prototrophic) are detectable
Disomic
a + c + e +
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Either
Or
Recombinant (v. rare)
However consider crossing-over, e.g. :-
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a + c + + f
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a b + d e +
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a b + d e +
+ b + d + f
Every disomic product is homozygous for at least one recessive marker and will be undetected

Fig. 1. Diagram to show how methods using multiply-marked chromosomes are biased to detect agents which interfere with chromosome pairing.

aneuploid gametes produced by the treated parent have a reasonably good chance of being "rescued" by fusion with an aneuploid gamete of a complementary type from the tester strain. As for the sex chromosome aneuploids, various types of exceptional offspring are recognized and distinguished by unusual inheritance of genetic markers. In a Drosophila system developed by Craymer (10) and Foureman (13) the inviability resulting from genetic imbalance is also turned to advantage. In this system males carrying an X;Y translocation are mated to chromosomally normal females. Only exceptional progeny (some arising from nondisjunction) are viable and so the effect of treatment on nondisjunction frequency can be monitored.

Tests using Drosophila have an important role to play. One advantage, unlike fungi, is that they are sensitive to colchicine (20,30). The existence of a number of meiotic mutants with different spontaneous frequencies of aneuploidy will also enable a further test of the observation that induction is proportional to the underlying spontaneous frequency.

In the mouse the available genetic methods and potential future developments have been comprehensively reviewed by Russell (this Volume), and so there is no need to go into details here. It is sufficient to note that there are several genetic methods available. Russell (31) has developed a sex chromosome anomaly test, but unfortunately this is limited by the availability of suitable markers, so that only chromosome loss can be detected in most crosses (9). Cattanach et al. (9) have developed a method, using Robertsonian translocations, of detecting autosomal aneuploidy in the mouse which is formally equivalent to Bateman's Drosophila method. This system is to be preferred to other tests in which both parents carry the rearrangement (22).

Cytological Methods

Cytological methods have been developed in several species of experimental mammals for detecting aneuploidy in germ cells, in first cleavage embryos, and in fetuses. These experimental approaches have been reviewed by Nijhoff and de Boer (27). All the cytological methods are, unfortunately, technically demanding and time-consuming, so that while they must play a central role in determining the precise effect of an experimental treatment, they cannot be used to screen a large number of chemical compounds. In labor-intensive methods the choice of which sex and which cell division stage are to be tested is an additional obstacle, because it is impractical to test the effect of treatments of a large number of chemicals on all stages in both sexes.

There is an urgent requirement to validate genetic testing methods in the mouse. They have considerable advantage over cytological methods being less demanding on skilled technical support and are therefore feasible for large-scale screening. There is a danger that too much reliance will be placed on microbial methods if a mammalian system is not developed.

THE CHOICE OF CHEMICALS TO BE TESTED

The real difficulty in designing a testing and validation program lies in the choice of substances to be tested. While there are a limited number of compounds which have been thoroughly tested over a range of organisms, there are hundreds of substances which have given some indication that they could be inducers. There is, for example, a fairly widely held view that any substance which induces mitotic arrest is likely to be an inducer. In 1970 Barthelmess (2) listed over 120 chemicals which interfered with the cell division spindle.

There are likely to be several targets in the cell because the successful distribution of chromosomes to daughter cells requires the coordinated activity of many cellular functions. The possibility that there are several potential targets in the cell obviously increases the difficulty of identifying active chemicals; they are likely to form a heterogenous group.

When considering the design of tests on a particular compound it is important to keep an open mind on how they might act. Consider, for example, the effect of hormone fluctuation on aneuploid production. It is thought that the age effect on maternal aneuploidy is best explained by hormonal fluctuations at menopause and evidence from hemiovariectomized mice supports this view (7). However, the hypothesis that hormonal fluctuation increases aneuploidy by inducing nondisjunction cannot explain the following observations:

(a) The age effect is confined to aneuploids which have gained a chromosome (18), but age-related monosomics are expected if the age effect originates through nondisjunction.

(b) Early selection against monosomic zygotes is unlikely to explain the absence of this class because a similar age effect acting only on gains is found in single cell zygotes of aged mice (24). Any explanation for this based on nondisjunction, therefore, has to act before this stage, perhaps in formation of the oocyte itself. This difficulty has been recognized by Hansmann (17) who suggested that, following nondisjunction, there is a preferential segregation into the oocyte to form a disomic product.

(c) Drosophila females also show an age effect providing that egg laying is restricted so that the meiotic process is stalled (35). The effect here is to increase both gains and losses, but the increase of the two types does not occur coincidentally. Aging of the mature oocyte results in chromosomal gains, while aging of earlier stages increases chromosome losses. It is very difficult to explain this observation based on a nondisjunction hypothesis because selection against nullisomic eggs would have to occur at one stage but not the other.

When mouse or Drosophila oocytes are held in an environment of fluctuating hormone levels there is an effect on aneuploid frequency. By what mechanism could hormones have this effect? The observations discussed by Chandley (this Volume) show that hormones appear to affect the cell division spindle. There is also considerable evidence that hormones affect chromosome replication. Thus it has been shown that fluctuation of estrogen levels initiates DNA synthesis in cells of the uterus (33), and that this stimulation occurs following exposure to physiological levels of the hormone (29). There are fluctuations in the responsiveness of the cells to estrogen and these appear to be under the control of the ovary (29). In menopausal women there are unusually long episodes of unopposed high estrogen excretion (25). It is not impossible therefore that the age effect could be explained by an effect on chromosome replication rather than chromosome distribution. These possibilities are two radically different alternatives which require tests of different cell division stages.

It is also important to realize that compounds might increase aneuploidy via a novel mechanism. Two examples spring to mind. Maguire (23) reported aneuploid induction by ethylene glycol in maize which promotes equational division of the chromosomes at the first meiotic division. The antianxiety drug, diazepam, induces mitotic arrest by inhibiting centriolar separation rather than by affecting microtubular integrity (1).

The design of the screening program is largely constrained by the number of tests which can be carried out on higher organisms, which imposes great difficulties in selecting the test chemicals. It is difficult to reconcile two conflicting requirements. On the one hand we would like to know what proportion of all substances are test-organism specific; on the other hand, validation of a range of test systems requires the selection of compounds which are likely to act both in meiotic and mitotic systems and in higher and lower eukaryotes. Selection of compounds on the basis that they have been shown to be positive in many organisms will tend to eliminate those which might act specifically. There are some interesting possibilities in this category. For example, dimethylsulfoxide has given positive results in Sordaria, yeast, and Drosophila (all meiotic systems), but it does not increase aneuploidy in mitotic systems or in Neurospora which is a meiotic system. Methylbenzimidazole carbonate and ethylene glycol are two other examples of compounds which might be test-organism specific.

The immediate priority will be to test a small number of selected compounds over a range of test organisms to establish which are useful methods for detecting induction. At some future date a differential screening protocol would seem appropriate in which a large number of suspected chemicals are screened in the more experimentally convenient systems and a selection of these chosen to be tested further using higher organisms. A coordinated program of testing is required to validate available screening procedures and to find out whether or not there is a potential environmental hazard from aneuploidy inducers.

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DISCUSSION

HANSMANN: I would like to raise one point that has not been covered during this meeting. One purpose of this Symposium is to cover the possibility that environmental hazards may increase the frequency of trisomic offspring in humans. We have only dealt with the problem of how environmental hazards may increase the frequency of nondisjunction. However, we have to deal with the problem that environmental hazards or environmental chemicals may alter selection during gestation. This could also be a problem that might increase the frequency of trisomic offspring.

ROLE OF ANEUPLOIDY IN EARLY AND LATE STAGES OF NEOPLASTIC PROGRESSION OF SYRIAN HAMSTER EMBRYO CELLS IN CULTURE

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INTRODUCTION

There is increasing evidence in support of the somatic mutation theory of carcinogenesis (2,6,8,12-15,21,36,49,50,56,57,60,70). The recent demonstrations of the involvement of point mutations in the activation of the ras oncogenes (49,60), the associations of chromosomal rearrangements with proto-oncogenes (50,70), and the amplification of oncogenes (36,56,57) in certain tumors add to other lines of evidence, such as the demonstration of DNA as a critical target in neoplastic transformation (6,8) and the correlation between mutagenesis and carcinogenesis (2,8,12,13) with most chemicals (see Ref. 8, 41, and 51 for discussion of the arguments against the somatic mutation theory).

For the past several years our laboratory has been interested in chemicals which are possible exceptions to the correlation between mutagenesis and carcinogenesis (13,15). We have contended that it is necessary to explain these exceptional chemicals in order to completely understand the role of genetic events in neoplastic development. During the course of these studies, we observed that certain chemical carcinogens, although inactive in specific-locus mutational assays, were effective inducers of aneuploidy. These chemicals induced an early stage of the neoplastic progression of cells in culture. Therefore, some of the previous exceptions to the correlation between mutagenesis and carcinogenesis can be explained if the definition of mutagenesis includes aneuploidy and other chromosomal mutations. Our data suggest that aneuploidy induction by certain chemicals is mechanistically involved in an early step in neoplastic development. In addition, we have found that numerical chromosome changes are involved in the later stages of neoplastic progression as well. In this review we will present these observations and discuss the possible significance of aneuploidy in neoplastic development.

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SYRIAN HAMSTER EMBRYO CELL TRANSFORMATION SYSTEM

For our studies we have used an experimental model in which both mutagenesis and carcinogenesis can be measured concomitantly (4,15). Early passage Syrian hamster embryo cells, when grown in culture and exposed to a wide variety of known chemical carcinogens, display morphological alterations in discrete colonies 1-7 days after carcinogen treatment (3,18,26, 28). The number of morphologically transformed colonies per total surviving colonies can be used as a quantitative measure of the carcinogen effect (18,26,28). These morphologically altered colonies are not neoplastic when first detected, but if the cells are grown further in culture they will progressively acquire other phenotypic alterations, such as an indefinite lifespan (called "immortality"), anchorage-independent growth, and ultimately tumorigenicity when injected into syngeneic hamsters or nude mice (3,15). Early, phenotypically altered cells, although not tumorigenic, have an increased propensity to become neoplastic and therefore are termed preneoplastic cells (7,15). Thus, we have developed a multistep model for studying different, qualitatively distinct stages in the neoplastic development of these cells. Early changes (i.e., morphological transformation and immortalization), as well as late stages (i.e., anchorage independence and tumorigenicity), can be studied and quantitated. Intermediate or preneoplastic populations can be isolated and characterized and their progression to the neoplastic state can be monitored.

In addition to being able to study different stages of neoplastic progression with Syrian hamster embryos, one can also measure induction of gene mutations at 2 genetic loci (4,5). Mutations in the Na^+/K^+ ATPase gene are measured by resistance to ouabain, an inhibitor of this enzyme, and mutations of the hypoxanthine phosphoribosyl transferase (HPRT) gene are detected by the ability of cells to grow in the presence of 6-thioguanine (TG), a toxic substance if phosphorylated by HPRT (5). By monitoring induction of ouabain-resistant (Oua^R) and TG-resistant (TG^r) cells concomitantly with cell transformation, one can obtain a direct comparison in the same target cells of mutagenic and carcinogenic potential of a chemical.

CELL TRANSFORMATION CAN OCCUR IN THE ABSENCE OF DETECTABLE GENE MUTATIONS

Using the Syrian hamster embryo system, we have compared the ability of a number of chemicals to induce gene mutations (TG^r and Oua^R) and morphological transformation, the first step in a multistep process of neoplastic transformation of these cells. We observed that chemicals which have mutagenic activity in this system are active transforming agents (4, 10,15). However, a number of chemicals (Tab. 1) induce cell transformation

Tab. 1. Chemicals that induce cell transformation but are reported as nonmutagenic.

Arsenicals*
Asbestos*
5-Azacytidine*
Benzene
Colcemid*
Diethylstilbestrol*
17β-Estradiol*
Ethionine*
Sodium bisulfite
Vincristine sulfate*

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* Gene mutation and cell transformation were measured concomitantly in the same cells.

Tab. 2. Types of genetic changes.

Gene mutations (point mutations, frameshift mutations, deletion and insertion mutations).

Gene duplication or amplification (increased copies of a gene).

Chromosome aberrations (translocations, inversions, and deletions).

Aneuploidy (abnormal numbers of chromosomes).

in the absence of detectable mutations in these two specific genetic loci (14,15).

As listed in Tab. 2, genetic changes can be subdivided into 4 categories. The absence of mutagenic activity at the HPRT or Na$^+$/K$^+$ ATPase loci indicates that a chemical does not induce gene mutations and certain chromosomal mutations. These two gene markers detect point mutations, deletion mutations, frameshift mutations, and rearrangements which result in gene inactivation (9,13). Although gene mutations and certain chromosomal mutations are not involved in the transforming activity of these chemicals, other genetic changes, either gene amplification or aneuploidy, may play a role in their transforming activity. In fact, as discussed below, our evidence indicates that aneuploidy induction is involved in the mechanisms of several of these agents. In addition, some of these chemicals (particularly 5-azacytidine and ethionine) may act by altering DNA methylation (34).

Chemicals Which Induce Both Aneuploidy and Cell Transformation

In the course of our studies, we have observed that a number of chemicals which induce aneuploidy induce morphological transformation (Tab. 3), suggesting that this early stage in the neoplastic development of these cells can be initiated by these chemicals.

The first chemical we examined was diethylstilbestrol (DES) which we observed could induce morphological and neoplastic transformation of Syrian hamster embryo cells in the absence of detectable gene mutations at two genetic loci (11). We have extensively studied the mechanism of action of DES in this experimental model and have concluded that the induction of aneuploidy by DES is mechanistically involved in its transforming activity (62).

We have found that although a natural estrogen, 17β-estradiol (E$_2$), is similar to DES in its transforming activity, transforming activity and estrogenic activity do not correlate for a number of DES-related compounds (40). Furthermore, Syrian hamster embryo cells have no detectable estrogen

Tab. 3. Aneuploidy-inducing chemicals that induce cell transformation.

Asbestos
Benzene
Colcemid
Diethylstilbestrol
17β-Estradiol
Vincristine sulfate

receptors and are not stimulated to grow by estrogens. Based on these observations, the estrogenic activity of these chemicals does not appear to be involved in their transforming ability (40). Rather, we propose that DES and 17β-estradiol have the ability to induce heritable alterations in cells which lead to the tumorigenic state. This transforming activity in conjunction with estrogen-stimulated cell proliferation may, in combination, result in hormonal carcinogenesis (40).

In an attempt to understand the mechanism of DES-induced cell transformation, we have examined the ability of DES to induce a variety of genetic changes in the Syrian hamster embryo cells. We have shown that DES induces cell transformation without causing gene mutations, unscheduled DNA synthesis, sister chromatid exchanges, or structural chromosome aberrations (11,62). However, DES is an effective inducer of aneuploidy, possibly via nondisjunction (62). Both chromosome losses and gains are induced by DES at doses which are nontoxic but transforming (Fig. 1). The importance of aneuploidy induction in DES-induced transformation is suggested from the following observations: (a) DES induces aneuploidy and cell transformation with parallel dose-response curves (Fig. 2); (b) aneuploidy induction correlates with the ability of DES-related compounds to induce cell transformation; (c) cell-cycle specificities of aneuploidy induction and cell transformation by DES indicate that cells in mitosis are most sensitive; and (d) neoplastic cell lines induced by DES are aneuploid with a near-diploid modal chromosome number (Ref. 14, and unpubl. data).

In collaboration with Dr. Robert Tucker of Johns Hopkins University, we have examined the effects of DES on microtubule organization in Syrian

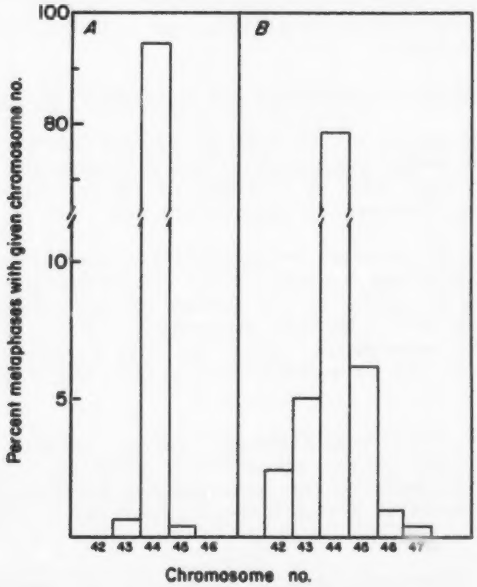

Fig. 1. Distribution of the chromosome number per metaphase in the near-diploid region for control (A) and DES-treated (B) cultures. Cultures were treated for 48 hr and the chromosome number was determined. For control cultures, 1,300 metaphases were examined, and for DES-treated cultures (1 μg/ml), 500 metaphases were examined. Reproduced from Tsutsui et al. (62).

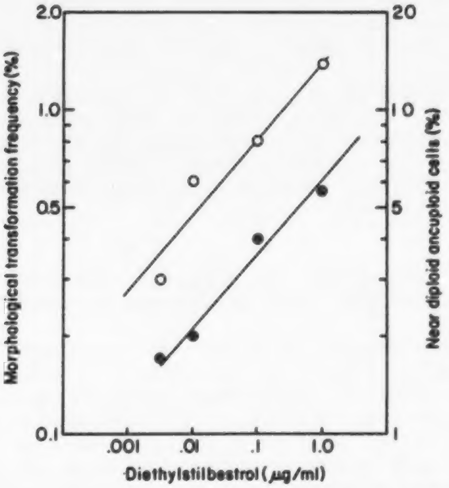

Fig. 2. Dose-response curves for DES-induced aneuploidy and morphological transformation. Cultures were synchronized and treated with varying concentrations of DES during the mitotic phase. The percentages of aneuploid (near-diploid) metaphases (o) and morphologically transformed cells (●) were determined. Reproduced from Tsutsui et al. (62).

hamster embryo cells. We found that concentrations of DES which cause aneuploidy also produce abnormal or arrested mitotic spindles by inhibiting microtubule polymerization from the centrosome (66). These studies provide a biochemical mechanism by which DES may disrupt the spindle apparatus and produce aneuploidy that results in cell transformation.

17β-Estradiol is similar to DES in its ability to induce transformation, although slightly higher doses are required. Like DES, E_2 also fails to induce gene mutations but induces nondisjunction (65). The dose-response curves for cell transformation and aneuploidy induction by E_2 are similar. These results indicate that both estrogens induce cell transformation by causing numerical chromosome aberrations.

Both DES and E_2 at high doses arrest cells in mitosis indicating that they are mitotic inhibitors. Therefore, it was of interest to determine whether cell transformation could be induced by other mitotic inhibitors, such as Colcemid and vincristine sulfate, which are also known inducers of aneuploidy. Treatment of the cells with Colcemid results in morphological and neoplastic transformation of the cells (63). Cell transformation is induced with doses which are noncytotoxic and below the concentrations necessary to cause mitotic inhibition of the cells. Higher doses of Colcemid result in mitotic inhibition of the cells and a significant loss of colony-forming ability, but no increase in morphological transformation. Treatment of the cells with transforming doses of Colcemid does not result in any measurable induction of gene mutations or structural chromosome aberrations; however, numerical chromosome changes are observed. The dose-response curves for Colcemid-induced morphological transformation and aneuploidy induction are similar (63). The shape of the dose-response curves and DES- and Colcemid-induced transformation and aneuploidy are also similar. Furthermore, induction of both endpoints occurs at doses of DES or Colcemid which do not increase the mitotic index. We interpret this finding to indicate that aneuploidy occurs at doses which cause chromosome segregation errors without affecting the rate of mitosis. Vincristine sulfate also induces morphological transformation and aneuploidy in Syrian hamster embryo cells with parallel dose-response curves (64).

These studies clearly show that specific chemicals which produce aneuploidy are also able to induce cell transformation. The lack of other genetic activities of these chemicals supports the hypothesis that this early stage in neoplastic transformation is induced by numerical chromosome changes. The next question we addressed was whether a nonrandom karyotypic change is involved.

TRISOMY OF CHROMOSOME 11: NONRANDOM KARYOTYPIC CHANGE IN IMMORTAL SYRIAN HAMSTER CELL LINES TRANSFORMED BY ASBESTOS

Further support for a role of aneuploidy in the early stages of cell transformation comes from our studies of a group of substances distinctly different from the microtubule blockers discussed above. Recently, we demonstrated that asbestos and other mineral dusts induce morphological and neoplastic transformation of Syrian hamster embryo cells in the absence of any measurable induction of gene mutation (32,42). It was also observed that asbestos at transforming doses induces significant increases in numerical chromosome changes, including aneuploidy and polyploidy, but was only weakly clastogenic (42). Since asbestos did not induce a great deal of nonspecific DNA damage, these findings prompted us to study the chromosome profiles in asbestos-induced, transformed Syrian hamster embryo cell lines in order to determine if any nonrandom chromosome changes are associated with the early stages of cell transformation.

We examined the acute cytogenetic effects of asbestos treatment of Syrian hamster embryo cells in culture to understand the mechanism of the transforming effects of this important environmental agent (42). The most pronounced effect of asbestos treatment was a change in chromosome number. Dose- and time-dependent increases were observed in the number of heteroploid cells with either a near-diploid or near-tetraploid or tetraploid chromosome number. Dose-dependent increases in the number of the cells with micronuclei or binuclei were also observed in the asbestos-treated cells. We have hypothesized that the numerical chromosome changes observed in our studies are due to physical and/or chemical interactions between the mineral fibers and chromosomes or the cytoskeleton protein(s) responsible for the movement of chromosomes (42). Hesterberg and Barrett (33) also obtained cytological evidence that asbestos induces significant increases in mitotic abnormalities; in some of the abnormal mitoses, asbestos fibers were observed in close contact with lagging chromosomes.

We attempted to identify any nonrandom karyotype changes in the preneoplastic stage of the asbestos-induced neoplastic transformation (46). Our findings were that 6 out of 8 immortal cell lines at the earliest passages examined had only numerical changes as a common abnormality. The remaining two cell lines contained both numerical and structural abnormalities. All 6 of the former cell lines contained a trisomy of chromosome 11, which at the earliest passage examined was the sole karyotypic change in some of these cell lines (Fig. 3). These findings strongly indicate that trisomy 11 plays an important role in the asbestos-induced cell transformation, at least in some cell lines. The two cell lines without trisomy of chromosome 11 had both structural and numerical changes not involving chromosome 11. In most of the cell lines, additional chromosome changes occurred in later passages; trisomy of chromosome 8 was most frequently observed (in 4 cases).

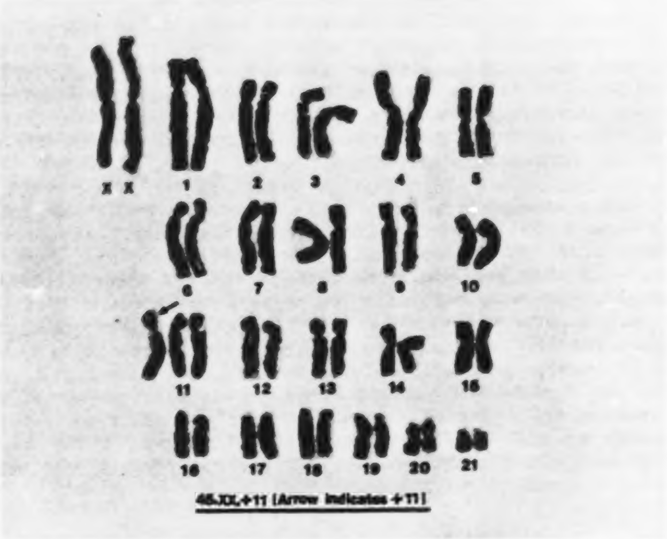

Fig. 3. G-banded karyotype showing trisomy of chromosome 11 in the ASB-10W cell line. Arrow indicates the extra chromosome. Reproduced from Oshimura and Barrett (43).

Our findings can be compared with those of others who have analyzed chemically transformed Syrian hamster embryo cell lines. Sachs and coworkers and later Benedict et al. reported that an increase in the number of chromosome 11 relative to other chromosomes was important in the tumorigenicity of these cells (16,20,68,69). Our results are partially consistent with this hypothesis in that an important role for trisomy of chromosome 11 is indicated. However, our data do not completely support the "balance theory" for tumorigenicity (20,68,69), since we have observed that cells with a trisomy of chromosome 11 are immortal and putatively preneoplastic, but not tumorigenic. Thus, the excess of "expressor genes" over "suppressor genes" may be necessary but not sufficient for tumorigenicity. This hypothesis would be consistent with the results of Sachs and coworkers (20,68,69), who observed that a change in the balance of these genes altered the tumorigenicity of revertant tumor cells. In this case, we assume that these cells had an additional genetic change which has not occurred in our asbestos-induced lines at the passages examined. In the cells with this additional alteration, the balance of chromosome 11 may control tumorigenicity. This hypothesis is consistent with the notion that different oncogenes act cooperatively to induce neoplastic transformation (38,52).

We also observed that some cell lines do not have a detectable alteration, numerical or structural, in chromosome 11. Thus, additional pathways for neoplastic development are suggested which is supported by the observation that multiple oncogenes can result in the same phenotypic alterations of cells in culture (19,37). The failure of DiPaolo et al. (27,48) to find specific chromosomal changes in Syrian hamster embryo cells may relate to this complexity.

DOUBLE NONDISJUNCTION DURING KARYOTYPIC PROGRESSION OF CHEMICALLY INDUCED SYRIAN HAMSTER EMBRYO CELL LINES

Having shown that aneuploidy was important in the early stages of neoplastic progression of Syrian hamster embryo cells by DES, Colcemid, asbestos, and other chemicals, we next examined the karyotypic evolution of these cells (43). We observed in 3 of 15 cell lines examined that the pattern of karyotypic evolution was consistent with the involvement of double nondisjunction (43), i.e., the loss of a normal chromosome and gain of the abnormal chromosome.

The postulated pathways of chromosomal evolution for these 3 cell lines are shown in Fig. 4. The common feature of each of these karyotypic progressions is the occurrence of double nondisjunction. The clearest example of this phenomenon is seen in the ASB-410 cell line. At an early passage (p13) this cell line has a translocation, t(13;21), and has lost the normal chromosome 13. At a later passage (p16) the translocated chromosome, der(21), is duplicated and in a few of the cells the normal chromosome 21 is also lost. Therefore, in this cell line 2 and, in some cases, 3 nondisjunction events have occurred. A similar mechanism must be invoked to explain the observed karyotype of DES-28 cells at late passage (p24), although we did not have intermediate passages of the cells to analyze. Double nondisjunction could account for the loss of the normal chromosome

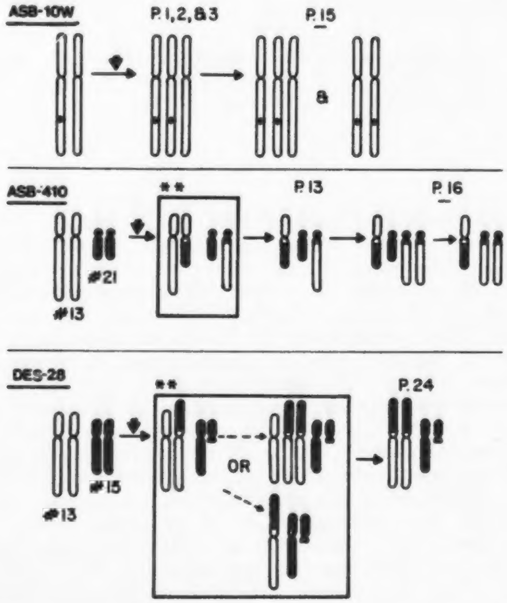

Fig. 4. Possible sequences of double nondisjunction as a karyotypic progression in 3 cell lines are shown. Thick arrows indicate the time of carcinogen treatment. Long arrows indicate the sequence of chromosome change which occurred. *: A possible submicroscopic change of DNA or unknown change different from the parental chromosome. **: Probable chromosome changes which had taken place; these changes were not observed. p = passage of given cell lines. Reproduced from Oshimura and Barrett (43) with permission.

13 and the duplication of the translocated chromosome in these cells. As shown in Fig. 4, either sequence of these two changes could have occurred.

An interesting example of double nondisjunction is the postulated karyotypic progression of ASB-10W cells. This clone was isolated following exposure of Syrian hamster embryo cells to asbestos. The only karyotypic alteration of this clone was a trisomy of chromosome 11, which presumably arose by nondisjunction after asbestos treatment. A pure population of cells with the trisomy was observed repeatedly upon karyotyping of cells at the first three passages after cloning. However, at a late passage apparently normal diploid cells appeared in the culture, which we propose resulted from a second nondisjunction of one chromosome 11 reverting the cells from trisomy 11 to disomy 11. Double nondisjunction provides one possible mechanism by which apparently normal diploid cells in a tumor may arise. If this is the case, cells originally trisomic for a chromosome may evolve a normal karyotype which has no apparent karyotypic alteration, but which may have arisen by 2 karyotypic changes, i.e., double nondisjunction. It is not known, however, whether the two apparently normal chromosomes have a submicroscopic alteration. If this were the case, double nondisjunction would result in an initial duplication of the chromosomes with this change and then a loss of the normal chromosome. If the gene with this alteration is regulated by a trans-acting mechanism from an allele on the normal chromosome, the initial nondisjunction event as well as the double nondisjunction could be important in escaping this regulation. Furthermore, double nondisjunction could result in changes in gene dosage and expression of recessive mutations.

Several investigators have recently examined the c-*myc* gene in murine T-cell leukemias (1,24,35,55,67). These tumors frequently are trisomic for chromosome 15 (35,55). In a few of these tumor-derived cells, a rearranged c-*myc* gene, caused by insertion of retrovirus-like sequences (23), is observed (1,35,67). Since this rearrangement is not found in all the T-cell leukemias, its significance in these tumors is still unknown (67). However, it provides a molecular probe to study nondisjunction in the karyotypic evolution of these cells. In the early passages of a tumor-derived cell line, both the rearranged and the germ-line sequences of the c-*myc* gene are observed (67). The results suggest that the cells with a trisomy of chromosome 15 have two copies of the chromosome 15 with the rearranged c-*myc* and one copy of the normal chromosome. A subline of these cells (TIKAUT), which still has a trisomy of chromosome 15 and is karyotypically indistinguishable from the parental cell, has only the rearranged c-*myc* sequences. Thus, the trisomy 15 cells must have undergone double nondisjunction, losing the normal chromosome 15 and duplicating the chromosome 15 with the rearranged c-*myc* sequences (67). Further, Cory et al. (24) have recently examined the high and low tumorigenic, TIKAUT-normal T_6 fibroblast hybrids. Low tumorigenic hybrids contained both normal and rearranged sequences. High tumorigenic segregants amplified the number of the tumor-derived and reduced the number of the normal, parent-derived chromosome 15s. It was suggested from the evidence that double nondisjunction occurred concurrently with the loss of the nonrearranged normal chromosome 15 and the gain of the abnormal 15 (35).

Recently, Cavenee et al. (22) proposed that retinoblastoma in individuals predisposed to this tumor develops after a recessive mutation becomes homozygous or hemizygous. By studying cloned DNA sequences of human chromosome 13 with restriction endonuclease polymorphisms, they proposed that double nondisjunction may be one mechanism responsible for the homozygosity of this change.

Thus, these studies as well as our own indicate that double nondisjunction is a recurrent event in karyotypic evolution of neoplastic cells.

One of the consequences of this is that cells with apparently normal karyotypes can evolve. This may obscure the role of karyotypic changes in the development of these tumors.

NONRANDOM LOSS OF CHROMOSOME 15 IN SYRIAN HAMSTER TUMORS INDUCED BY v-Ha-*ras* PLUS v-*myc* ONCOGENES

Nonrandom chromosome rearrangements, observed in a variety of human and animal tumors (53,55), are associated in some cases with enhanced expression or deregulation of cellular oncogenes (36,39). Recently, it was shown that normal, diploid rodent cells are neoplastically transformed following transfection with 2 cooperating oncogenes, for example *myc* plus *ras* (38,52,61). However, the number of steps necessary to convert a normal cell into a malignant cell is unknown. If activation of 2 oncogenes is sufficient for tumorigenicity, then tumors derived from diploid cells transformed by the transfected oncogenes may remain diploid or have only random chromosome alterations. We have performed cytogenetic analyses of tumors formed following transfection of Syrian hamster embryo cells with either v-Ha-*ras* plus v-*myc* DNAs or polyoma DNA alone (45). Whereas polyoma-induced, tumor-derived cells were diploid, tumors induced by v-Ha-

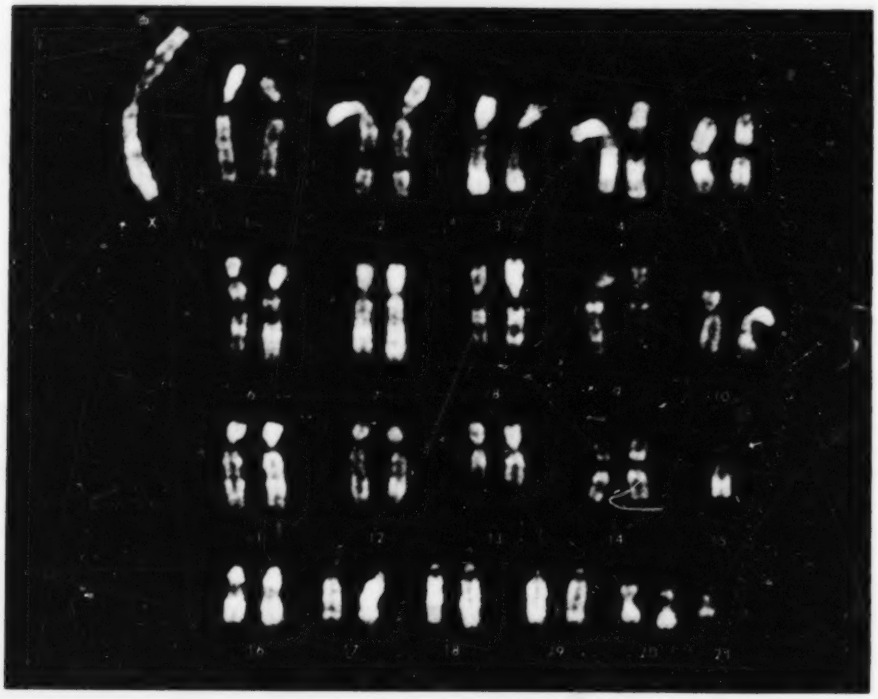

Fig. 5. A banded karyotype of a cell with 42,X,-X,-15 karyotype in a v-Ha-*ras* plus v-*myc*-induced tumor. Two arrows indicate missing chromosomes X and 15. The monosomy 15 was a common chromosome change observed in all the cells analyzed.

ras plus v-myc oncogenes were monoclonal and had a nonrandom chromosome change, i.e., monosomy of chromosome 15 (Fig. 5) (45). Thus, an additional change, loss of chromosome 15, is required or advantageous for tumorigenicity induced by v-Ha-ras plus v-myc oncongenes. These results suggest that neoplastic progression of normal, diploid cells requires more than 2 steps under certain conditions.

Nonrandom chromosome loss has been observed in a variety of human and animal tumors and transformed cells (25,31,53,55,71). Pathak et al. (47) observed that 15 of 18 virus (simian adeno 7, chicken embryo lethalorphan, herpes simplex, and simian papova viruses) transformed Syrian hamster cell lines were monosomic for chromosome 15. This is similar to our findings with ras/myc tumors.

We interpret our findings to indicate that the loss of a specific chromosome results in the loss of a cellular gene which affects a phenotypic change necessary or advantageous for neoplastic development or the deletion of trans-acting cellular sequences (39) which regulate the expression of either the v-Ha-ras or v-myc genes. This additional change occurred either spontaneously in these cells or may have been induced by transfection with v-Ha-ras plus v-myc DNAs. Several authors have also proposed that the dosage of cellular factors regulates the expression of tumorigenicity in viral and chemically transformed cells (22,54,72). Chromosome loss from hybrids between normal and malignant cells (17,59) results in the expression of tumorigenicity. In contrast to cell hybridization studies which suggest that tumorigenicity is a recessive phenotype (17,59), DNA transfection studies have indicated that tumorigenesis is due to dominantly acting genetic elements (58). Our findings that chromosome loss can occur following DNA transfection and tumor formation may help to resolve these seemingly disparate conclusions. The oncogene-transfection system may be useful for understanding chromosomal control in carcinogenesis and the role of cellular factors in the regulation of the expression of oncogenes and tumorigenicity.

CONCLUSIONS

In the course of our studies on the biological significance of karyotypic changes in Syrian hamster embryo cells treated with chemical carcinogens or transfected with oncogenes, we observed the following lines of evidence which suggest an important role for aneuploidy induction in carcinogenesis:

(a) Several chemicals, which fail to induce gene mutations, induce morphological transformation and aneuploidy (e.g., DES, Colcemid, asbestos), suggesting a primary role in cell transformation for at least certain chemical carcinogens.

(b) A nonrandom gain of chromosome 11 was observed in 6 out of 8 asbestos-induced cell lines at early passages. This finding indicates that +11 plays a role in the early stages of immortalization and neoplastic progression.

(c) Karyotypic evolution of 2 asbestos-induced and 1 DES-induced cell lines involved "double nondisjunction," i.e., duplication of an abnormal chromosome and concurrent loss of the normal chromosome. These results indicate that double nondisjunction is a mechanism for karyotypic progression during neoplastic development.

(d) Early-passage Syrian hamster embryo cells cotransfected with v-Ha-ras and v-myc DNAs formed tumors in either nude mice or newborn hamsters. The cells from all 6 tumors had a nonrandom loss of a chromosome 15. Thus, neoplastic transformation in these cells requires chromosome loss in addition to activation of myc and ras oncogenes.

These observations suggest that aneuploidy is associated with an early event of carcinogenesis in some cases as well as a later change in others. The possible biological roles of numerical chromosome changes are: (a) change in gene dosage, including increase in the product of a dominant acting gene, decrease in the product of a gene, and change in rate of expression of a gene (dosage compensation effect); (b) change in gene balance (gene dose vs other genes or alleles); (c) phenotypic expression of a recessive mutation; and (d) change in genetic stability, including increased mutagen sensitivity, change in DNA repair, and change in target size for mutations. (See Ref. 29 for further discussion.) Trisomy of chromosome 11 and monosomy of chromosome 15 may be examples of gene-dosage phenomena involving an increase in the product of a presumptive gene in the former and a decrease in the latter. Double nondisjunction may be important in either changing gene balance or phenotypic expression of a recessive mutation (30).

Future studies with molecular probes will be necessary to elucidate further the biological role of aneuploidy in cancer. Numerical chromosome changes may affect neoplastic progression by a number of distinctly different mechanisms, which explains why aneuploidy is associated with a primary event of carcinogenesis as well as with later stages. The role of nonrandom aneuploidy in human and animal tumors has been reviewed previously by Oshimura and Barrett (44) and by Evans (this Volume).

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ETIOLOGY AND MECHANISMS OF ANEUPLOIDY: A SYNOPSIS

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INTRODUCTION

During the last few years, there has been increasing concern about the possibility that environmentally induced aneuploidy may pose a threat to human health. This concern is of particular interest to the U.S. Environmental Protection Agency (EPA) and other agencies because of regulatory responsibilities in the area of mutagenesis (Voytek, this Volume). Consequently, the National Institute of Environmental Health Sciences (NIEHS) and the Reproductive Effects Assessment Group (REAG) of the EPA sponsored workshops on the subject in 1978 and 1983, respectively. The NIEHS workshop focused on test systems for the detection of aneuploidy; its content is published in Volume 31 of the journal Environmental Health Perspectives. Topics considered at the REAG workshop include the adverse effects of aneuploidy on human health, tests for detecting chemicals that induce aneuploidy, and mechanisms of aneuploidy. The REAG workshop has been summarized in a review by Dellarco et al. (3); although problems as difficult as those involved in assessing risks posed to human health by aneuploidy cannot be resolved in a short workshop, the workshop served to focus attention on the subject and to provide the impetus for this Symposium.

Like the earlier workshops, this Symposium has not resolved the difficult scientific and policy questions that underlie the concern about aneuploidy. In view of the complexity of the problem, no one could have expected that it would. The Symposium has, however, brought together information from diverse lines of investigation that relate to aneuploidy as a concern for human health. These lines of investigation concern the following problems:

(a) The etiology of aneuploidy in humans.

(b) Quantification of the impact of aneuploidy on human health.

(c) Cytological and biochemical processes in mitosis and meiosis.

(d) Perturbations of meiotic and mitotic processes that can lead to aneuploidy.

(e) Methods for detecting agents that induce aneuploidy.

(f) Prospects for assessing health risks associated with the induction of aneuploidy.

In bringing together information on these subjects, the Symposium has also served to point out where there are significant gaps of knowledge. This is a necessary step in directing research toward important problems in the field and, ultimately, in improving our ability to formulate appropriate strategies for minimizing the impact of aneuploidy on human health.

HUMAN ASPECTS

The human aspects of aneuploidy include measuring the incidence of aneuploidy in humans, identifying genetic and environmental risk factors for its occurrence, and assessing its etiology and impact. The impact, of course, includes social and economic components that can be estimated, although not easily (Conley; Edel and Edel, this Volume), as well as more intangible elements in human suffering that are extremely difficult to quantify but are nevertheless very real (Edel and Edel, this Volume). Although these problems are complex and controversial, there is one conclusion about which there is little disagreement--aneuploidy is a significant problem for human health. Moreover, the magnitude of the problem would argue that a substantial effort to minimize the occurrence and impact of aneuploidy is warranted. It is a curious inconsistency that aneuploidy has received relatively little emphasis in mutation research and genetic toxicology when it has such a clear adverse effect on human well-being.

Measuring frequencies of aneuploidy in human populations is complicated by such factors as the means of collecting data and the structure of the population with respect to age and other variables. Nevertheless, a few numbers can illustrate the magnitude of the problem. About 0.3% of all newborn infants are aneuploid (Ref. 1,4,5; and Hassold, this Volume), including such disorders as Down, Patau, Klinefelter, and Turner syndromes. Moreover, about 4 to 6% of infant deaths are associated with aneuploidy (5). In clinically less severe cases, aneuploidy can still contribute to learning and behavioral disorders, disorders of sexual development, and infertility (5). Besides its effects in newborns, aneuploidy contributes significantly to fetal deaths. It has been estimated that at least 4 to 5% of recognized pregnancies involve aneuploidy (Ref. 1,5; and Hassold, this Volume) and that 30 to 35% of all spontaneous abortuses are aneuploid (Ref. 5; and Hassold, this Volume). Although there are difficulties in determining frequencies of aneuploidy in human gametes, estimates of 1.6% and 5% have been made for sperm on the basis of cytogenetic studies of hamster eggs fertilized by human sperm (Martin, this Volume). Direct examination of meiotic chromosome preparations from humans has not provided independent confirmation of the high rates of aneuploidy in human gametes (Hulten et al., this Volume), but more extensive studies will be required to resolve inconsistencies in measured frequencies of aneuploidy in germ cells.

The frequencies of aneuploidy among human conceptions, abortuses, or newborns point to aneuploidy as an important concern for human health. Although such numbers reveal a high frequency of aneuploidy in humans relative to that in other species (Ref. 1; and Bond; Hulten et al., this Volume), they do not provide a clear view of the origins of human aneuploidy or of the effects of selection with respect to the frequency of aneuploid gametes, zygotes, and later stages of development. I would therefore like to comment on several factors that should be considered in analyzing the origins of human aneuploidy and its implications for health.

The Cellular Origin

Aneuploidy can originate in either mitosis or meiosis. Meiotic aneuploidy is important for health because fertilization involving aneuploid gametes leads to aneuploid conceptuses that die prenatally or develop into aneuploid offspring. Consequently, much of the concern about aneuploidy has focused on meiotic aneuploidy.

The emphasis on meiosis does not mean that mitotic aneuploidy is unimportant for health. Aneuploidy arising in embryologic development may contribute to the occurrence of mosaicism for euploid and aneuploid chromosomal constitutions (5). Although most mosaics probably arise by chromosome loss from trisomic zygotes or embryos, a mitotic origin of aneuploids from normal diploid cells may make a small contribution to clinically significant cases of aneuploidy and may contribute to cryptic mosaicism that can be a risk factor for aneuploidy in the next generation (7). Moreover, aneuploidy in the somatic cells of children or adults has been implicated in the development of several cancers, as have chromosomal rearrangements and mitotic recombination (Evans, this Volume). An association has also been reported between mitotic aneuploidy and malignant transformation in mammalian cells in vitro (Barrett et al., this Volume).

In cases of meiotic aneuploidy, it is often possible to trace the parental origin of the abnormality and to distinguish errors in meiosis 1 from errors in meiosis 2. Information on the etiology of aneuploidy can contribute to an understanding of the distribution of risk of aneuploidy in human populations and can also be important for identifying targets that should be represented in test systems for aneuploidy, because tests should include the most sensitive stages of gametogenesis (Russell, this Volume). Information on chromosomal origins may even have bearing on the phenotypic expression of trisomic conditions, because data from mice suggest the possibility that severity may vary depending on the specific parental origin of the extra chromosomal material (Searle and Beechey, this Volume).

Technical advances have made it easier to detect whether particular cases of human aneuploidy are of paternal or maternal origin. In addition to using the Xg antigens of red blood cells for analyzing some cases of X-chromosomal aneuploidy, one can now analyze many cases of autosomal aneuploidy by means of heteromorphisms in chromosome banding and polymorphisms in lengths of restriction fragments (Hassold, this Volume). In combination, these methods should make it generally possible to distinguish maternal from paternal origin and errors in the first meiotic division from errors in the second meiotic division. Analysis of restriction fragment length polymorphisms (RFLPs) is a particularly valuable technique for analyzing human aneuploidy and promises to be even more so when RFLPs have been characterized in the centromeric regions of the chromosomes (Hassold, this Volume).

Some generalizations about the origin of aneuploidy in humans are now possible. Data for trisomy 21 reveal that the extra chromosome is maternal in origin in about 70 to 80% of cases (Hook, this Volume). Although the majority of cases arise by nondisjunction in maternal meiosis 1 (Hassold, this Volume), errors in spermatogenesis or in maternal meiosis 2 are not insignificant, in that they account for about one-third of cases. Like trisomy 21, other trisomies seem to arise most commonly in maternal meiosis 1; there are differences among chromosomes, however, in frequencies of trisomy that are detected (e.g., trisomy 16 is most common in abortuses) and in the proportion of trisomies of paternal origin (Hassold, this Volume).

Unlike trisomies, monosomy for the human X chromosome does not arise principally through meiotic nondisjunction. Rather, most cases seem to involve sex chromosome loss by mechanisms that do not involve the formation of a reciprocal trisomic product (Ref. 1; and Hassold, this Volume). The chromosome that is lost is of paternal origin in the majority of cases

(Hassold, this Volume). Since human trisomies and X-chromosomal monosomy generally arise by different mechanisms, they can differ markedly from one another with respect to risk factors. It is therefore necessary to achieve an understanding of all mechanisms that contribute to human aneuploidy, so that all possible targets for the induction of aneuploidy are considered in the development of test systems for the detection of induced aneuploidy.

The Age Effect

It has been recognized for some time that the risk of trisomy 21 increases with maternal age. Specifically, the incidence of Down syndrome increases slowly with age of mothers between ages 19 and 30 yr, and after age 30 the rate undergoes a more pronounced increase (Chandley; Hook, this Volume). The maternal-age distribution of Down syndrome suggests the possibility of there being age-dependent and age-independent components of human aneuploidy; if so, one could estimate that 35 to 50% of all Down syndrome is age-dependent (Hook, this Volume). Most, but not all, other trisomies also exhibit a maternal-age effect but its magnitude differs among chromosomes (Chandley; Hook; Kline and Stein, this Volume). Unlike trisomy, X-chromosomal monosomy exhibits no increase with maternal age and may actually undergo a slight decrease (Hook, this Volume); this difference in age dependence is not surprising in view of the difference in mechanism underlying these two kinds of aneuploidy (Hassold, this Volume). Similarly, the frequency of XYY, which results from nondisjunction in paternal meiosis 2, exhibits no clear parental-age association (Hook, this Volume).

Although the phenomenon of a maternal-age effect in the incidence of trisomy is well known, its basis is not. The notion that the age effect is fully ascribable to increased probability of nondisjunction in aged oocytes now seems too simplistic. For example, it has been suggested that the size of the remaining store of oocytes, which is an indication of physiological age, is more important in determining the probability of maternal nondisjunction than is simple chronological age (Chandley, this Volume). Moreover, there is a maternal age effect in the incidence of Down syndrome even if one only considers cases in which the extra chromosome is paternal in origin (Ref. 4; and Hook, this Volume). It therefore seems possible that reduced selection against trisomics in older mothers also contributes to the age effect. If so, the evidence from spontaneous abortions would suggest that this selective influence occurs very early--prior to the recognition of pregnancy (Hook, this Volume).

The age effect is of obvious importance because of its usefulness in identifying prospective parents who are at increased risk and in evaluating the desirability of prenatal diagnosis for aneuploidy. It is not at all clear, however, how the age effect relates to potential environmental risk factors. With our present state of knowledge, we can barely identify those agents that are most apt to pose a threat of inducing aneuploidy in humans. Once we have a better understanding of that problem, it will be of interest to explore whether endogenous factors, such as age, that contribute to a high incidence of spontaneous aneuploidy also lead to increased risk of induced aneuploidy. Obtaining such information will depend on having appropriate animal models. With respect to the age effect, there is still insufficient evidence on the similarity of age effects reported in other mammalian species (Chandley; Hansmann et al., this Volume) to that in humans.

Genetic Risk Factors

Although predispositions toward aneuploidy in humans are not well characterized yet, cryptic parental mosaicism would seem to be a strong risk factor (Ref. 7; and Warburton, this Volume); its contribution to the total incidence of aneuploidy may be small, however, because of the relative infrequency of its occurrence. Even for de novo nondisjunction, there is no reason to believe that the risk of aneuploidy is borne evenly by all genotypes. There is evidence of high frequencies of aneuploid sperm in men who carry certain chromosomal rearrangements (Martin, this Volume). The presence of certain unusual nucleolus organizing regions has also been described as a factor that predisposes to nondisjunction (Hassold; Warburton, this Volume). More subtle genetic contributors to aneuploidy are also likely. For example, couples who have had a trisomic child are found to have an increased risk of trisomy in subsequent pregnancies (Kline and Stein; Warburton, this Volume). Such predisposition toward trisomy could occur by either an increased rate of nondisjunction or decreased selection against trisomy early in pregnancy. An increased tendency toward mitotic aneuploidy in parental lymphocyte cultures has also been described as a possible risk factor for having trisomic offspring, but the data are inconsistent (Warburton, this Volume). Several other possible genetic risk factors have been suggested by Warburton. If genetic factors that increase the risk of aneuploidy can be clearly delineated, it could be possible to identify individuals at high risk for counseling purposes. It would also be important to know whether individuals at high risk of spontaneous aneuploidy are at high risk of induced aneuploidy.

Environmental Risk Factors

In view of the adverse effects of aneuploidy on human health, it is of obvious importance to identify any environmental agents that cause increases in its incidence. All current evidence on possible associations between environmental agents and the incidence of aneuploidy in humans involves great uncertainty (Kline and Stein, this Volume). Several studies have explored possible associations between the incidence of trisomy and maternal exposure to ionizing radiation. While one cannot preclude some association between radiation exposure and aneuploidy, the results are inconsistent and do not provide unequivocal evidence for such an association (Ref. 5; and Kline and Stein, this Volume). There are data that suggest a lower incidence of trisomy associated with maternal cigarette smoking, especially in young mothers, but the reason for the inverse correlation is unclear (Kline and Stein, this Volume). Other factors that have been suggested as possible contributors to aneuploidy include oral contraceptives, spermicides, and caffeine (Kline and Stein, this Volume). A dose-dependent relationship is reported between the frequency of triploid abortuses and maternal caffeine exposure, measured as coffee consumption (Kline and Stein, this Volume), but the result needs to be confirmed. In the cases of the other agents, the data are negative, inconsistent, or inconclusive.

More complex associations, such as those suggested by differences in the apparent frequencies of aneuploidy among countries, places of residence, ethnic origins, or lifestyles (Ref. 3,4; and Hassold; Kline and Stein, this Volume) are very difficult to interpret with respect to specific causative factors. Temporal trends in the incidence of Down syndrome have been too inconsistent to permit any conclusions regarding causes (5).

Although not based on aneuploid progeny, there is evidence from studies of hamster eggs fertilized by human sperm for the induction of aneuploidy in the sperm of cancer patients receiving radiotherapy (Martin, this Volume). Nevertheless, no clear association has been demonstrated between aneuploidy and any environmental exposure under more usual circumstances (Ref. 5; and Kline and Stein, this Volume). It should be noted, however, that the data do not have sufficient statistical power to preclude modest effects (Kline and Stein, this Volume).

The lack of epidemiologic evidence for induced aneuploidy in humans is not surprising. The inconclusive results may relate as much to the insensitivity of the methods as to an actual absence of effects. The situation seems parallel to the lack of unequivocal evidence for the induction of transmissible gene mutations in human germ cells by mutagens. Because of confounding variables, inadequate sample sizes, and technical limitations, epidemiologic approaches for the detection of any genetic alteration in human germ cells are not particularly sensitive. Clear experimental data from mice, however, leave little doubt about the capacity of ionizing radiation and certain chemicals to induce gene mutations and chromosome aberrations in the germ cells of mammals, and there is no reason to expect humans to be an exception. Methods for studying the induction of aneuploidy in the germ cells of laboratory animals are not as advanced as methods for studying other classes of genetic damage, but some data on aneuploidy induced in mammalian germ cells are available. Our experience with germ cell mutagenesis in mammals and the fact that chemically induced aneuploidy has been demonstrated in various experimental organisms argue that we should not derive great comfort from the lack of clear associations between particular environmental agents and the incidence of human aneuploidy.

Because of the great importance of identifying risk factors for aneuploidy, epidemiologic approaches should be pursued vigorously. Means of monitoring rates of aneuploidy in human populations include cytogenetic screening of livebirths and abortuses, analysis of results from prenatal cytogenetic diagnosis, phenotypic surveys of populations, and monitoring the ratio of de novo translocations to trisomies among cases of Down syndrome (5). The relative precision and costs of these methods have been discussed in a review article by Hook (5). However, the insensitivity of epidemiologic methods and the desirability ot identifying potential inducers of aneuploidy before the occurrence of human exposure make it essential that we develop experimental test systems that can predict effects in humans. Developing such tests depends on acquiring a sound understanding of the origins of human aneuploidy, and that understanding depends on an expanded database on the prevalence and genetic characterization of aneuploid gametes, abortuses, and livebirths in humans. Factors that should be taken into consideration in developing appropriate experimental models include the diversity of potential cellular targets for the induction of aneuploidy and differences in susceptibility among species, between sexes, between meiotic and mitotic divisions, among stages of gametogenesis, and among organisms of differing ages.

MECHANISMS

It is only through continued progress in research on mitotic and meiotic mechanisms that we can hope to identify the diversity of targets that exist for the induction of aneuploidy. For example, agents that interfere with the functions of tubulin, the principal protein of spindle fibers, are likely candidates for inducing aneuploidy (Hsu and Satya-Prakash, this Volume). Consistent with this expectation, such chemicals as colchicine and vinblastine are known to induce aneuploidy in several experimental organisms (Ref. 1; and Hsu and Satya-Prakash, this Volume). The problem, however, goes well beyond tubulin. Mitosis and meiosis are complex, regulated, dynamic processes and, as such, provide multiple targets that may cause aneuploidy if their functions are disrupted. In this respect, recent reports that the synthetic estrogen, diethylstilbestrol, selectively damages kinetochores and centrioles and thereby induces aneuploidy in cultured mammalian cells (Brinkley et al., this Volume) are of particular interest.

We should view mitotic and meiotic spindles as dynamic rather than static structures (McIntosh; Nicklas, this Volume). The fibers, which are composed of bundles of tubulin microtubules, are formed at the centrosomes—organizing centers that become the spindle poles (Brinkley; McIntosh, this Volume). Although much remains to be learned about the relative roles of centrosomes and kinetochores in the initiation and organization of spindle microtubules (Ref. 6; and De Brabander et al.; McIntosh, this Volume), it has become clear that some of the spindle fibers from a given pole interact with fibers that extend from the opposite spindle pole, whereas others are attached to chromosomal kinetochores (McIntosh, this Volume). There is evidence that the initial associations of the chromosomes with the spindle fibers are often incorrect but that cellular processes rectify the inappropriate associations (Nicklas, this Volume).

Recent studies in yeast reveal the existence of short sequences of DNA, organized into a distinctive chromatin structure, in the centromeric regions of the chromosomes. These sequences are involved in stabilizing the behavior of the chromosomes in mitosis and meiosis (Yeh and Bloom, this Volume). It will be of interest to explore similarities and differences between these findings in yeast and the nature of kinetochore structure and function in mammalian cells (Brinkley et al., this Volume).

Whether chromosomal or interpolar, the spindle fibers emanating from a given pole exhibit the same polarity. Fibers from opposite poles differ in polarity and can interact with one another. The interaction at anaphase seems to involve a sliding of the microtubules with respect to one another, thereby elongating the spindle and increasing the distance between the poles. Besides this interaction between spindle fibers of opposite polarity, the fibers themselves elongate at anaphase through addition of tubulin subunits (McIntosh, this Volume). While the interacting fibers elongate, the microtubules that are attached to kinetochores lose tubulin subunits and shorten at anaphase chromosome movement (McIntosh, this Volume).

These dynamic processes involve a rapid exchange of tubulin subunits between microtubules and the cellular pool of soluble tubulin. The process is highly regulated, and the rate of subunit exchange is reduced for kinetochore microtubules during metaphase and for interacting microtubules from opposite poles during anaphase (McIntosh, this Volume). Much remains to be learned about the controlled stabilization and destabilization of microtubules; the mechanisms involve a variety of specific microtubule-associated proteins, ATP-dependent and ATP-independent processes, and regulatory processes in which calcium ions seem to play an important role (Ref. 3,6; and De Brabander et al.; McIntosh, this Volume). It would therefore seem that there are a variety of cellular targets whose disruption could cause spindle malfunction (Liang and Brinkley, this Volume). Besides failures to form normal bipolar spindles (Hsu and Satya-Prakash, this Volume), alterations in kinetochore structure and function and in the regulated stabilization or destabilization of spindles are potential sources of aneuploidy (Nicklas, this Volume).

An awareness of the diversity of potential targets accentuates the complexity of attempting to identify all classes of agents that pose a risk of inducing aneuploidy in humans. Such an awareness is important, however, for developing relevant model systems for the study of induced aneuploidy. A functional testing capability will depend on the development of assays that respond to chemicals that affect spindle function and chromosome movement by any of several possible mechanisms. In this respect, we need to learn more about the extent to which components of the mitotic and meiotic processes have been conserved in evolution, because the similarity or dissimilarity of the targets among organisms has obvious implications for the validity of extrapolation among species. For example, the generality of DNA being the principal target of mutagens offers a reasonable rationale for extrapolation in other areas of genetic toxicology. A better understanding of mitotic and meiotic mechanisms in phylogenetically diverse organisms will be necessary for knowing whether we can regard experimental data on the induction of aneuploidy in fungi, plants, or Drosophila as being reasonably likely to predict similar effects in mammals. An increasing understanding of mitotic and meiotic mechanisms can therefore help to guide our efforts in test systems development.

It is not yet feasible to make quantitative risk assessments for aneuploidy because of insufficient knowledge about induced aneuploidy and limitations in our current test systems. However, the EPA and other government agencies are faced with the difficult task of having to quantify human health risks (Voytek, this Volume). We may therefore anticipate future efforts in basic research and test development that will move us toward a better capability for quantitatively predicting human risks. As we progress in that direction, we may also anticipate that the problem of multiple targets for the induction of aneuploidy will loom larger. The dose-response relationships for the induction of aneuploidy may be as diverse as the mechanisms of induction, making the problems of extrapolation among dosages and the likelihood of thresholds particularly problematic.

While recognizing that test systems in mitotic cells can be useful, we must keep in mind that much of the aneuploidy that causes adverse effects in humans is meiotic in origin. Some of the targets for the induction of aneuploidy are common to mitotic and meiotic divisions, so results on the induction of aneuploidy in mitotic cells may predict similar effects in meiotic cells. Some potential targets, however, are specific to meiosis, and it is therefore essential that model systems continue to be developed to measure frequencies of meiotic aneuploidy (Russell, this Volume).

Studies in diverse organisms have provided evidence of meiosis-specific targets for induced aneuploidy. For example, a segment of DNA that affects meiosis but not mitosis has been identified in a chromosomal region flanking a centromere in yeast (Yeh and Bloom, this Volume). In plants, there are DNA sequences that are widely distributed among the chromosomes and that are referred to as zygotene DNA because they replicate primarily in zygotene rather than in the premeiotic S-phase; the replication and transcription of zygotene DNA seems to be required for normal synapsis (Stern and Hotta, this Volume). Failure of synapsis, moreover, is of concern because both nonconjunction and nondisjunction can lead to aneuploidy (Ref. 1; and Moses et al., this Volume). In principle, any of several meiotic errors can lead to aneuploidy. These include: (a) nonconjunction of homologs, (b) nondisjunction of homologs, (c) precocious centromere division in meiosis 1, (d) failure of chromatids to separate at meiosis 2, (e) extra chromosome replication, and (f) chromosome loss (1).

Synapsis and recombination provide important potential targets for the induction of aneuploidy in meiosis (Russell, this Volume). For example, the alkylating agent cyclophosphamide causes synaptic failure, fragmentation of the synaptonemal complex, and aneuploidy in mammalian germ cells (Moses et al., this Volume). With respect to recombination, factors that inhibit normal crossing-over and those that stimulate recombination may both have the potential to interfere with the proper disjunction of homologs (Mason and Resnick, this Volume). We must therefore strive for a better understanding of synapsis, recombination, and chiasma interference and of the relationships among them (Grell; Moens; Moses et al., this Volume). Besides the mechanics of these processes, their timing and the hormonal regulation of mammalian gametogenesis can be of critical importance in the development of normal haploid gametes (Ref. 4; and Grell; Hansmann et al., this Volume). The foundation for a better understanding of how environmental agents may stimulate rates of meiotic aneuploidy lies in mechanistic studies on the major events of meiosis--replication, synapsis, recombination, and segregation--and on how they are regulated and relate to one another (Ref. 1; and Green; Grell; Hansmann et al.; Moses et al., this Volume).

TEST SYSTEMS

An integrated approach to genetic toxicology requires effective, sensitive assay systems for the three principal classes of mutational damage that are relevant for human health: (a) gene mutations, (b) structural chromosome aberrations, and (c) numerical chromosome aberrations. For technical and historical reasons, the development of effective genetic toxicology tests for numerical chromosome aberrations has lagged behind that for other genetic effects, despite the obvious importance of aneuploidy for human health. The increasing concern about aneuploidy during the last few years, however, has stimulated interest in aneuploidy tests and has provided the impetus for further research and analysis on the subject.

Having tests for the detection of aneuploidy is particularly important because the mechanisms underlying its origin are very different from those of other classes of genetic damage, in which DNA is the principal target. Tests for DNA damage, gene mutations, and structural chromosome aberrations may therefore not detect some inducers of aneuploidy. Agents that have been reported to induce aneuploidy include various mitotic arrestants, hormones, alkylating agents, antibiotics, and other chemicals (Ref. 1,3,4; and Dellarco et al.; Waters et al.; Liang and Brinkley, this Volume). Although some conventional mutagens like methyl methanesulfonate or N-methyl-N'-nitro-N-nitrosoguanidine are included among the agents that induce aneuploidy, it is plain that the list is not a standard list of mutagens. The possibility that the induction of aneuploidy is not associated with that of other genetic effects argues that the development of assay systems for aneuploidy be given a high priority. Such assays should have the capacity for detecting agents that induce aneuploidy by any of several mechanisms, including direct interaction with DNA and chromosomes, alteration of meiotic pairing or recombination, disruption of the function of tubulin or microtubules, or interaction with spindle function by other, more indirect, mechanisms (1).

Various tests for aneuploidy are being developed, and it is important that the more promising assays be used to expand the database on the induction of aneuploidy by chemicals. Aneuploidy can be detected in organisms as diverse as yeasts, mycelial fungi, plants, insects, and mammals. As in other areas of genetic toxicology, there is a troublesome inverse correlation between the presumed relevance of a given assay for human health and the practicality or expense of performing it (Bond, this Volume). Other areas of genetic toxicology are dependent on sensitive, rapid, inexpensive short-term tests for screening compounds and identifying potential hazards and tests in higher organisms for a more thorough characterization of genetic risks. It would seem prudent to proceed similarly in the development of tests for aneuploidy; and promising methods that are under development in both lower and higher eukaryotes should be encouraged. In this respect, we can particularly look forward to continued progress with tests for mitotic and meiotic aneuploidy in fungi, tests for aneuploidy arising in gametogenesis in Drosophila, and genetic and cytogenetic mammalian tests (Russell, this Volume). In view of uncertainties about the relative sensitivity of different animal models, comparative data for several mammalian species would be particularly valuable (Hansmann et al., this Volume).

Factors that should be considered in selecting model compounds for comparative studies in diverse assays include results in the current genetic toxicology database and likely effects on particular cellular targets (Liang and Brinkley, this Volume). In any case, a considerably expanded database will be required for evaluating the performance of particular assays. Validation of assays in a strict sense is impossible because there is no relevant reference point in humans. Data on concordance or discordance among assays can, however, give some sense of the likely predictive value of assays for humans and of the legitimacy of extrapolation among species.

Limitations of the current database prevent definitive conclusions from comparisons among assays (Dellarco et al.; de Serres; Waters et al., this Volume), but there are several issues that comparative studies will ultimately have to resolve. These include: (a) the extent to which assays in lower eukaryotes predict effects in mammals (Bond; Dellarco et al.; de Serres; Liang and Brinkley; Waters et al., this Volume); (b) the extent to which assays that detect mitotic aneuploidy also predict the induction of meiotic aneuploidy (Dellarco et al.; Liang and Brinkley; Waters et al., this Volume); (c) the extent to which high spontaneous frequencies of aneuploidy, such as that observed in humans, are correlated with high susceptibility to induced aneuploidy (Bond, this Volume); (d) similarities and differences between the responses of male and female germ cells to the induction of aneuploidy (Ref. 3; and Liang and Brinkley, this Volume); (e) dose-response relationships for induced aneuploidy, including such issues as whether agents that induce aneuploidy are often effective over only a narrow range of dosages (Brinkley et al., this Volume) and whether there are commonly no-effect thresholds at low dosages (3); and (f) the concordance or discordance of aneuploidy tests with tests for other kinds of genetic damage (Dellarco et al.; Waters et al., this Volume).

Besides evaluating results from an expanded literature on induced aneuploidy, it will ultimately be beneficial to perform collaborative studies to explore interlaboratory variability in the performance of some of the more promising assays (de Serres, this Volume). Questions of standardization in testing and criteria for an acceptable test will also have to be addressed for aneuploidy testing, as they are now being debated for other kinds of genetic toxicology tests. For the short term, however, the thrust should be toward the development of sensitive, efficient, and reproducible assays and toward the use of these assays for expanding the database in this underemphasized area of genetic toxicology.

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